

FOCAL ADHESION SIGNALING IN BREAST CANCER TREATMENT

Yafeng Ma

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Focal adhesion signaling in breast cancer treatment

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Front cover and back cover:

Along the River During the Ch'ing-ming Festival
Court painters, Ch'ing Dynasty (1644-1911)
Handscroll, ink and colors on silk, 35.6 x 1152.8 cm

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CHAPTER 1

GENERAL INTRODUCTION:

**Focal adhesion and chemokine receptor-mediated
signaling in breast cancer progression**

Metastatic breast cancer

Breast cancer is the most common type of cancer among women. A report of the ENCR (European Network of Cancer Registries) showed that the Netherlands had a highest incidence rate of breast cancer ($91.6/10^5$), leading to the third highest mortality ($26/10^5$) in the year 2000 (www.enchr.com.fr/breast-factsheets.pdf). Typically, primary breast cancer can be removed by surgery in combination with chemotherapy and radiation therapy. However, metastases are very difficult to treat and most breast cancer patients eventually die due to drug resistant distant metastasis. Therefore, it is important to understand the underlying molecular mechanisms by which metastasis occurs, and design target-specific drugs to prevent metastasis formation and overcome the drug resistance of disseminated breast cancer.

Metastases are formed by transmission of malignant cells from a primary tumor site to the distant target organs throughout the body. The process from primary tumor to metastasis occurs in several steps including: **1.** transformation of normal cells into cancer (stem) cells; **2.** proliferation of cancer cells and formation of primary tumor in microenvironments; **3.** detachment of tumor cells from primary lesion, which generally involves an epithelial-mesenchymal transition-like process of tumor cells; **4.** invasion of tumor cells into the microenvironment and degradation and restructuring of the extracellular matrix; **5.** intravasation of escaping tumor cells into blood or lymphatic vessels; **6.** escaping the (innate) immune system surveillance and homing of viable tumor cells to target organs; **7.** extravasation of tumor cells from the vascular system in distant target organs; **8.** survival and proliferation of metastasizing tumor cells in distant organs; **9.** development of new blood vessels, *i.e.* angiogenesis, which supply nutrients to support macroscopic metastatic outgrowth as shown in Figure 1.

Cell adhesion, migration and survival

Cell adhesion of epithelial cells occurs at cell-cell adherence junctions through E-cadherin molecules and at cell-extracellular matrix (cell-ECM) adhesions through integrin receptors. As mentioned above, typically, epithelial-derived tumors may metastasize when cell-cell adhesions are lost during the epithelial-mesenchymal transition (EMT). This allows cells to migrate, invade and eventually metastasize. Tumor cells require external triggers to initiate EMT and mediate cell migration. In the tumor microenvironment, various growth factors, chemokines and cytokines either derived from stromal cells or secreted by tumor cells are present. These will stimulate their respective receptors on tumor cells and consequently activate the cell migration program described below. Although cell migration and invasion may take place by single cell migration and collective cell migration strategies [1], here I will only discuss the steps of single cell migration, which is the most critical step in the dissemination of tumor cells [2,3]. Cell migration requires a sequential set of events, including plasma membrane protrusion and

adhesion formation at the leading edge of lamellipodia to the substrate, contractile force generation, translocation of cell body, and release of the rear edge of cell [4].

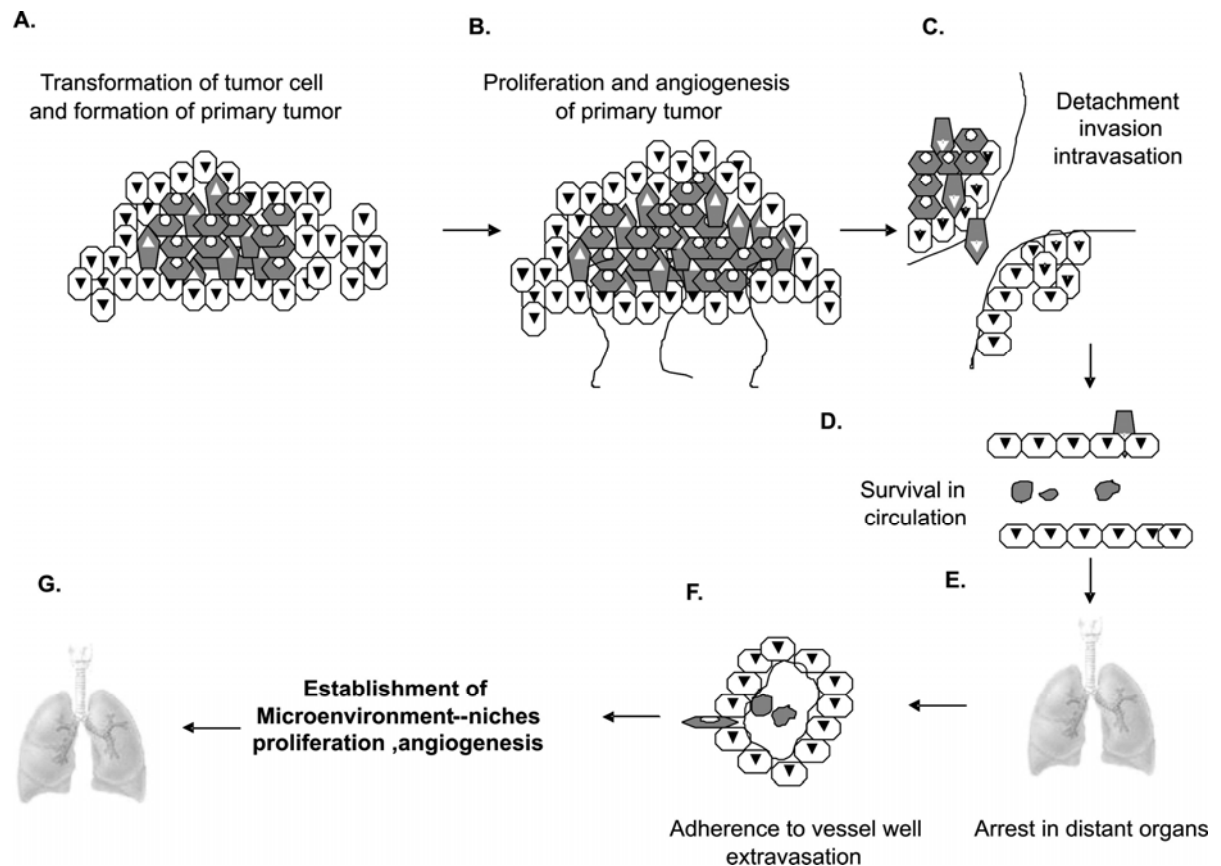


Figure 1. The multiple steps in metastasis formation. A. cellular transformation at primary sites. Growth of neoplastic cells must be progressive, with nutrients initially supplied by simple diffusion to expand tumor mass. B. tumor cell proliferation and extensive vascularization. The secretion of angiogenic factors establishes a capillary network to supply nutrients from surrounding tissues. Chemokines and receptors are essential for cell recruitment in tumor microenvironment. C. tumor cell detachment from primary malignant sites, invasion and penetration to circulation. D. survival of disseminated cells in vascular environment. E. transportation through the body and arrest in distant organs. In this step, chemokines provide chemotaxis clues to target organs. F. cell attachment on the vessel wall and extravasation to nearby tissues. G. proliferation of tumor cells and angiogenesis in metastatic niches.

Integrin-mediated adhesion is crucial for cell migration process. At the lamellipodia, focal complexes (FCs) are formed and mature into larger focal adhesions (FAs). FAs are the sites which link adhesion receptors and proteoglycans to the actin cytoskeleton and they consist of scaffold molecules, GTPases, and enzymes such as kinases, phosphatases, proteases and lipases. FAs are not only anchor sites but also sensors for mechanical and biochemical signaling [5,6]. The assembly and disassembly of FAs occur during cell migration. The FCs which are regulated by Rac and Cdc42 at the leading edges of migrating cells, exert traction force on ECM to relocate cell body. The majority of these small FCs undergo fast turnover, while few mature into FAs. The latter are regulated by Rho activity and form just behind the leading edges of cells. FAs are responsible for

tensile force generation which allows movement of the cell body. FAs either disassemble underneath the moving cell body or change in shape and form fibrillar adhesion for ECM modification. FAs at the rear of cells maintain cell spreading and ultimately dissociate to release the rear of the cells [6]. All these steps are tightly regulated by different scaffolding and signaling molecules at the focal adhesion sites, including integrin, focal adhesion kinase (FAK), c-Src, paxillin, talin and vinculin [6-10]. Tyrosine, serine and threonine phosphorylations of various FA-associated proteins are major signaling events at FA sites to control the dynamics of these structures and regulate cell migration. For example, tyrosine phosphorylation of FAK creates docking sites for binding of Src-homology (SH) domain containing proteins, e.g. SH2/SH3-containing proteins and thereby regulates the activation of protein kinases, such as Src [7]. Scaffold functions are executed by the adaptor proteins, including for example paxillin and p130Cas. Tyrosine phosphorylation of these scaffolds allows recruitment of downstream effectors and enhances specificity of the signaling.

Integrin-mediated cell migration requires pulling forces generated by cytoskeletal contraction of the actin cytoskeleton. This is mediated by the actin/myosin network within the cell and the ‘anchors’ derived at FAs through the integrin-ECM interactions. Actin filaments are cross-linked by myosin complexes, resulting in bundling as well as contraction of these actin fibers [11]. The contractile forces are regulated by myosin light chain (MLC) phosphorylation via MLC kinase and Rho-associated kinase (ROCK). ROCK controls the phosphorylation of myosin and inhibits myosin phosphatase [12]. MLC phosphorylation is a biochemical marker for the status of tension in the cell [13]. Given the essential role of the actin cytoskeleton dynamics in cell migration, there is also a need for regulation of the actin network. Briefly, this is mediated by Rho GTPases family. Activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) stimulates actin polymerization via the Arp2/3 complex (a seven-subunit protein regulating actin cytoskeleton) at the leading edge, leading to plasma membrane protrusion and extension of lamellipodia [14]. RhoA, another small GTPase protein, promotes the formation of contractile actin-myosin filaments and this is essential for cell contractility and FA assembly and disassembly [4,15]. Besides the Rho GTPases, the actin cytoskeleton organization is also regulated by calcium-dependent proteases, i.e. the calpains. Calpains are a large family of calcium-dependent cysteine proteases that cleave myofibril or cytoskeleton associated proteins to disassemble the FA complexes [7, 8].

Signaling at the cell-ECM contacts not only regulates cell migration processes but also controls the survival of cells. In normal epithelial cells, loss of cell-ECM and cell-cell interactions causes the onset of apoptosis, also called *anoikis*. There is increasing evidence that the enhanced adhesion signaling in tumor cells at cell-ECM contact sites is important for the inhibition of *anoikis* in metastatic tumor cells [16]. Such an enhanced signaling allows the survival of tumor cells in both tumor microenvironment and the circulation. Given this paradigm, metastatic tumor cells would be relatively resistant to anticancer drugs. Indeed, inhibition of integrin-signaling in different tumor cells makes

them more susceptible to anticancer drug-induced apoptosis [17]. Drug-induced apoptosis is characterized with distinct cellular and biochemical features which include cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. The onset of these apoptotic events is mediated through the release of mitochondrial cytochrome c, activation of the apoptosome and generation of active caspase-9 and caspase-3. Survival signaling derived from integrin-mediated adhesions triggers the activation of survival pathways that inhibit the release of cytochrome c from mitochondria and prevent the onset of caspases [18]. These include the activation of phosphoinositide-3 kinase (PI3K)/AKT [19] as well as suppression of the pro-apoptotic activity of p53 [20]. Although the roles of some FA-associated proteins in the control of cell survival have been identified, the exact signaling pathways downstream from cell-ECM interactions that control apoptosis are not entirely clear, in particular under *in vivo* conditions.

Signaling pathways in cancer progression

Detrimental cancer progression can not occur without an increase in signal transduction that enhances proliferation, survival and metastasis. During cancer progression, many prominent molecules and signaling pathways are activated due to either overexpression of receptors for example members of the ErbB family, such as EGFR and ErbB2 in breast cancer, or loss of tumor suppressor genes such as the lipid phosphatase PTEN to enhance the PI3K/AKT pathway. It is beyond the scope of this introduction to discuss all the signaling pathways that are involved in cancer. In the context of this thesis, I will only detail the following concepts: **1.** the roles of growth factors and chemokines (and receptors) in breast cancer cell migration and metastasis; **2.** mitogen-activated protein kinases (MAPKs)-involved cell growth, cytoprotection and motility as well as the role of Fra-1, a member of MAPK downstream transcription factor activator protein-1 (AP-1), in cancer progression; **3.** survival and growth signaling mediated via phosphoinositide 3 kinase (PI3K)/protein kinase B (PKB, also named AKT). These pathways are mediated by FA complexes and we then mainly focus on **4.** FAK-related cell migration and survival in cancer progression; **5.** paxillin-associated migratory and survival signal.

Growth factor and chemokine receptors in cancer

Growth factor receptors

The ErbB family of protein tyrosine kinase receptors plays an important role in breast cancer progression and drug resistance. High expression of EGFR and ErbB2 [21], as well as another tyrosine kinase receptor c-Met [22,23] are associated in reduced breast cancer patient survival. Increased expression of these receptors promotes survival of breast tumor cells. EGFR is activated by epidermal growth factor (EGF) while c-Met by hepatocyte growth factor (HGF; also named scatter factor). EGF serves as not only a growth factor to facilitate cell survival and proliferation, but also a typical chemotactic growth factor that triggers directional lamellipodial protrusion towards concentration

gradient, formation of free barbed ends and actin polymerization at the leading edge in some cell lines. *Condeelis* and co-workers have systematically studied EGF-induced phenotypic changes and signaling pathways in living rat mammary adenocarcinoma MTLn3 cells using various approaches including advanced live cell microscopy [11-14]. EGF induces a PI3K-dependent cell protrusion, where Ras is required for PI3K activation and lamellipod protrusion while Rac1 for formation of adhesive structures [24]. RhoA/ROCK regulates the switching between Cdc42 and Rac1 at the leading edge [25]. Moreover, phospholipase C (PLC), LIM-domain kinase (LIMK)-cofillin, and PI3K-Wiskott Aldrich syndrome protein (N-WASP)-ARP2/3 are implicated in formation of barbed ends and lamellipodial protrusion upon EGF stimulation [26, 27]. Importantly, the EGFR signaling is essential for enhanced migration and metastasis of MTLn3 cells *in vivo* [28, 29]. HGF, which promotes the EMT process in various epithelial cell lines [30, 31], binds the tyrosine kinase receptor c-MET, thereby activates similar downstream signaling and promotes mitogenesis, cell motility and invasion [32].

Chemokine receptors

Chemokines (a subfamily of chemoattractive cytokine) and chemokine receptors (a subfamily of seven transmembrane G-protein coupled receptors), are implicated in cell directional migration and cancer metastasis [33,34]. Many chemokines and receptors are expressed by tumor cells and cells in the tumor microenvironment, including stroma cells (endothelial cells, fibroblasts) and leukocytes. Chemokines provide chemotaxis signals for tumor cells to target specific organs and attract leukocytes to tumor sites [35]. Accumulative evidence shows that chemokine receptor activation involves the following tumor-related processes [36]: providing the movement direction for migrating cells (chemo-attractive effect) via cytoskeleton reorganization; shaping the tumor microenvironment by cell recruitment and angiogenesis modulation; providing survival and proliferation signaling via downstream effectors, e.g. AKT and ERK1/2 (extracellular signal-regulated kinase), and transcription factors such as NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) [37]. The tumorigenic properties and implications of diverse chemokine receptors in cancer are listed in Table 1. Malignant cells exhibit aberrant expression of particular chemokines and receptors, notably the CXC chemokine receptor 4 (CXCR4, also named fusin), the C chemokine (C-C motif) receptor 7 (CCR7) and C chemokine (C-C motif) receptor 10 (CCR10) [38,39]. Various studies on breast, colon and prostate cancers have established that cancer cells express more CXCR4 than the corresponding normal epithelial tissue [40-44]. Downregulation of CXCR4 by microRNA or antagonists prevents tumor invasion and metastases *in vitro* and *in vivo* [45,46]. Moreover, CXCR4-Chemokine (C-X-C motif) ligand 12 (CXCL12) axis potentiates the crosstalk between tumor cells and the microenvironment and activates the intracellular activity of MAPKs and AKT [30-33]. However, the molecular mechanisms and roles of other chemokine receptors in breast cancer cell migration and tumor development remain largely unknown. The chemokine receptor CXCR3 promotes colon cancer metastasis to lymph node [47] and melanoma cell metastasis to lymph node [48]. Reduction of CXCR3 expression with antisense RNA or special neutralizing antibodies

against receptors suppresses metastasis [49]. However, so far, relatively little is known about the molecular mechanism and function of CXCR3 in breast tumor cell motility and invasion.

Table 1. Chemokine receptors in cancer research

	Tumorigenic properties	Implication in cancers
CXCR1/2	Angiogenesis, invasion, metastasis, growth, proliferation, MMP expression	Colorectal, lung, melanoma
CXCR3	Invasion, metastasis, growth, proliferation	Colorectal, melanoma
CXCR4	Angiogenesis, invasion, metastasis, growth, proliferation, MMP expression, DC recruitment	Breast, etc, 23 types
CXCR5	Invasion, metastasis, growth, proliferation	Carcinomas (pancreatic, colon, etc)
CXCR7	Growth survival	Breast, lung
CCR1/2	TAM recruitment, polarization, invasion, metastasis, angiogenesis, mmp expression	Breast, lung, prostate, etc
CCR3/5	TAM recruitment, invasion, metastasis, angiogenesis, MMP-19 expression	Breast ,cervical, etc
CCR4	TAM and T-cell recruitment, invasion, metastasis	Ovarian, Hodgkin's lymphoma, etc
CCR6	DC recruitment, proliferation, invasion, metastasis	Breast, colorectal, etc
CCR7,9,10,	Survival, invasion, metastasis	Breast, melanoma, etc
CX3CR1	Survival, invasion, metastasis	Prostate

Chemokines were classified for CXC, CC, C and CX3C, according to the position of cysteine in the N-terminal. The implications of chemokine receptors in cancers are briefly listed and edited from [39]. MMP, matrix metalloproteinase; DC, dentritic cells; TAM, tumor-associated macrophages.

Downstream signaling

In this thesis, the roles of several signaling pathways downstream of either growth factor or chemokine receptors have been studied in relation to breast tumor cell migration, metastasis and cell survival. To update all the present knowledge on the diversity of signaling pathways that are essential in tumor cell biological programs, I refer to several outstanding papers on these subjects [1, 4, 25, 35, 39, 50-55]. Here, I would like to briefly discuss the MAPKs, downstream AP-1 transcription factors with the focus on Fra-1, and the PI3K/AKT pathway in the link to the rest chapters. Furthermore, I will mainly describe the regulations and functions of two essential focal adhesion-associated proteins, FAK and paxillin, in more detail.

MAP kinase pathways

ERK, JNK and p38 signaling

Mitogen-activated protein kinase (MAPK) pathways involve evolutionarily conserved kinases that control cell growth, proliferation, differentiation, migration and apoptosis [56,57]. The MAPK family mainly includes the subfamilies of ERK, JNK and p38 kinases.

The activation of receptor tyrosine kinases triggers guanosine triphosphate (GTP) loading of Ras GTPase, which then recruits Raf kinases to the plasma membrane. Activated Raf stimulates MEK1 and MEK2 by phosphorylation on serines 218 and 222 in their activation loop. ERK1/2 is activated upon phosphorylation by MEK1/2. Consequently, active ERKs phosphorylate numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeleton proteins (such as paxillin, see further below), thus inducing various cellular biological outcomes. Most cancer-associated lesions have elevated Ras/Raf/MEK/ERK activity due to either increased expression of (mutant) growth factor receptors such as EGFR, ErbB2 or activated Ras [58-60]. Sustained ERK activation promotes phosphorylation and stabilization of early response genes, such as Fos, Jun, Myc and Egr-1, and regulates cell-cycle entry by controlling the expression of downstream cyclin-related kinases and inhibitors [61]. Although transient activation of ERK fails to promote cell differentiation or proliferation, it regulates cytoskeletal dynamics and promotes cell movement by myosin light chain kinase (MLCK) and Rac1 [62, 63].

Depending on specificity of cell type, JNK is activated by e.g. cytokines, UV radiation, growth factor deprivation or DNA-damaging agents [64-67]. There are three isoforms of JNK (JNK1, JNK2, JNK3) and the activation requires dual phosphorylation on tyrosine and threonine residues at a distinctive Thr-Pro-Tyr (TPY) motif. One major group of JNK substrates activated by stress response are apoptosis-related proteins Bcl-xl, Bim, Bmf and Bad [65, 68], modulating the mitochondrial and cytochrome c-mediated apoptotic pathway. The other group of substrates is transcription factors like c-Jun, Fra-1, ATF, Elk-1 and NFATc1, which regulate cell cycle arrest and gene expression of cell invasion related proteins, such as matrix metalloproteinases (MMPs) [69-71]. JNK activation by growth factor alters the transcriptional regulation of MMP9 through transcription factor AP-1 [72]. JNK also regulates cell migration via phosphorylation of cytoskeleton-associated proteins and scaffold protein substrates. For instance, ERK and JNK can phosphorylate paxillin [73], which will be further discussed later in this chapter and in the following chapters.

Another MAPK is p38, which is activated by stress and inflammatory cytokines. There are four isoforms of p38. Once activated, p38 proteins translocate from the cytosol to the nucleus where they phosphorylate serine/threonine residues of substrates. p38 pathway also plays a role in the regulation of apoptosis, cell cycle progression, growth and differentiation in a cell context-dependent manner [74].

The AP-1 transcription factor Fos-related antigen-1 (Fra-1)

Activator protein 1 (AP-1) is a transcription factor and this homodimeric or heterodimeric protein complex consists of the Jun, Fos and ATF (activating transcription factor) families. The Jun family includes c-Jun, JunB and JunD, while the Fos family consists of c-Fos, FosB, and fos-like antigen-1 and 2 (Fra-1 and Fra-2) [75]. Both homo- and

heterodimers of Jun protein can bind DNA directly, while Fos members require the interaction with Jun protein. Jun homodimers have little or no DNA binding activity in comparison with Jun/Fos heterodimers [76]. AP-1-binding response elements, for instance, TRE, CRE and ARF, interact with other proteins like p65 subunit of NK- κ B, CBP/p300, Smad-3 and -4, thereby controlling gene expression involved in proliferation/transformation, pro- and anti-apoptosis, cell cycle, migration and invasion [77-82]. AP-1 is activated via MAPK cascades by growth factors, cytokines, stress, UV, etc [83-86]. The activity and expression of Fra-1, Fra-2, c-Jun and JunB are particularly linked to the sustained activation of ERK1/2 or JNK pathway [83, 87].

Fra-1 expression levels are significantly enhanced in highly invasive cells [88,89] and correlate with the mesenchymal characteristics of epithelial tumors. Expression profiles of a panel of 27 human mammary cell lines [90] reveal a highly upregulated expression of Fra-1 in ‘fibroblast tumor’ cluster compared with ‘epithelial tumor’ cluster. It is the up-regulation of Fra-1 expression, rather than a mutational activation, that contributes to the growth and proliferation of human tumors. Fra-1 is regulated at transcription and post-translation level via a diversity of promoters, enhancers and signaling pathways, for example, AKT [91, 92], β -catenin/Tcf and Raf/MEK/ERK cascades [89-91] (see Figure 2). Fra-1 promotes cell survival and growth by the regulation of cell cycle entry. AP-1 regulates breast cancer cell growth via cyclins (cyclin D) and E2F factors [93]. Fra-1 controls cyclin A transcription in Ras-transformed thyroid cells [94] and cyclin D1 expression at G₀-G₁ cell cycle entry [95]. Fra-1 and JunB may actually antagonize cell cycle progression.

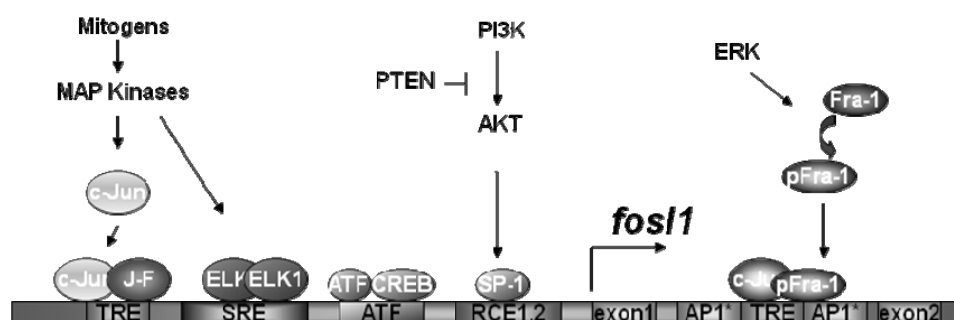


Figure 2. The regulation of Fra-1 expression. Various mitogens, cytokines, toxicants and carcinogens control the induction of Fra-1 via signal transduction pathways and protein interaction of cis-element and cognate trans-factors. Fra-1 gene expression is activated by the MAPK pathway. Fra-1 binds to its own promoter through trans-activators (Jun, Fos family and ELK1) interaction with cis-element at the TPA response element (TRE), serum response element (SRE) and ATF site in the *fosl-1* promoter. PI3K-AKT regulates *fosl-1* expression through the retinoblastoma control element (RCE). Fra-1 is phosphorylated and stabilized by ERK signaling. Fra-1 and cJun regulates *fosl-1* expression by binding the TRE site in the first intron. cJ = c-Jun, JF = JunD or Fra-2, AP-1* = AP-1-like site. Adapted from Young & Colbun, 2006 [96].

Fra-1 regulates cell motility and migration. This relates to the activation of the Rho-

ROCK pathway through most likely indirect modulating the function of β 1-integrin. Silencing of Fra-1 with siRNA leads to loss of cell polarity, motility and invasiveness, formation of stress fiber, and stabilization of focal adhesions [97]. Fra-1 also regulates the expression of several cancer progression and tumor cell migration-associated genes including for example CD44 and c-Met [98], as well as the expression of MMPs [99,100]. Particularly, Fra-1 enhances the motility and invasion of lung epithelial cells by inducing the activity of MMPs, in particular, MMP-2 and MMP-9 [100]. Fra-1 is also implicated to regulate vimentin during HA-RAS-induced EMT in human colon carcinoma cells [101]. Since growth factor signaling regulates Fra-1 expression and Fra-1 regulates cell migration, we wondered the relationship between FAK activity and Fra-1 expression in the context of breast tumor cell migration and anticancer drug sensitivity. This has not been investigated before and will be discussed in this thesis.

The PI3K/AKT signaling pathway

Receptor-mediated activation of Phosphoinositide 3-kinase (PI3K) originates from the recruitment of p85 subunit of PI3K via its SH2 domain to phosphorylated tyrosine residues in the intracellular domains of growth factor receptors. The p110 catalytic subunit of PI3K catalyzes the phosphorylation of the phosphatidylinositol-containing lipid PIP₂ at its 3-position resulting in increased levels of PIP₃ in the plasma membrane. AKT is recruited from the cytosol to the plasma membrane through intramolecular interaction with PIP₃ and PIP₂ where AKT undergoes conformational changes and becomes activated through phosphorylation on Thr308 and Ser473 by PDK and PKA [102]. The lipid phosphatase PTEN dephosphorylates PIP₃, thereby antagonizing the AKT activation.

AKT signaling is strongly implicated in diverse cancers, and deregulation of AKT function promotes cancer progression [103-105]. In addition, AKT regulates cell cycle through phosphorylation and cytoplasmic retention of the cell cycle inhibitors p21 and p27 as well as enhanced translation and stabilization of cyclin D1 [106]. AKT also influences cell survival and prevents apoptosis via enhancing glucose uptake and promoting the NF- κ B pathway which regulates some pro-survival genes and inhibits pro-apoptotic Bad and Bax [107]. Activated AKT also promotes MDM2 nuclear translocation, increases Bcl-2/Bcl-xl levels, and inhibits cytochrome c release from mitochondria [108]. There are three distinct isoforms of AKT (AKT1, AKT2 and AKT3), among which AKT1 and AKT2 show different functions in cell invasiveness and cancer development [109]. Generally, AKT1 regulates cell growth and survival signaling, while AKT2 regulates metabolic signaling. In fibroblasts, AKT1 promotes cell migration via phosphorylation of an actin-binding protein girdin and therefore forms stress fiber and lamellipodia [110]. In fibrosarcoma cells and mouse mammary epithelia cells, AKT enhances cell invasion via increased secretion of MMPs [111,112]. Remarkably, in cells where one AKT isoform stimulates motility, the other usually has a limited or even opposite role. AKT1 and AKT2 have distinct roles in Rac/Pak signaling, cell migration [113] and mammary

adenocarcinoma development in mouse mammary tumor virus (MMTV)-ErbB2/Neu and MMTV-polyoma middle t (PyMT) transgenic mice [109].

The focal adhesion-associated tyrosine kinase focal adhesion kinase (FAK)

FAK domains and interactions

Integrin clustering and activation of growth factor receptors recruit FAK and c-Src at FA sites and transmit adhesion-dependent and growth factor-induced signals into the cell interior. FAK is a multidomain non-receptor tyrosine kinase. The structure, phosphorylation sites and protein-protein interaction domains of FAK are shown in Figure 3. The N-terminal region of FAK contains a Band 4.1, ezrin, radixin and moesin homology domain (FERM). The FERM domain, particularly a basic surface exposed region in subdomain-2, negatively regulates the catalytic activity of FAK through an intramolecular interaction with FAK kinase domain. FERM interacts with integrins and growth factor receptors [114]. FAK, via its FERM domain, also binds the Arp2/3 complex to control actin assembly [115]. Alternatively, the FAK FERM domain binds to FIP200 (FAK-interacting protein of 200kDa), which confers an inhibitory effect on FAK catalytic kinase activity, thus negatively regulates FAK function [116]. On the other hand, in tumor progression, FAK enhances survival signals by binding to FIP200, consequently limiting FIP200-p53 interaction and suppressing p53 activity. FAK proline-rich regions (PRR1 and PRR2) bind Src-homology-3 (SH3) domain-containing proteins such as Crk-associated substrate (p130Cas), GTPase activating protein for Rho associated with FAK (GRAF) and the Arf-GTPase-activating protein 1 (ASAP1). The focal adhesion targeting (FAT) domain of FAK is c-terminally located and interacts with paxillin and talin, resulting in the focal contact localization of FAK [7].

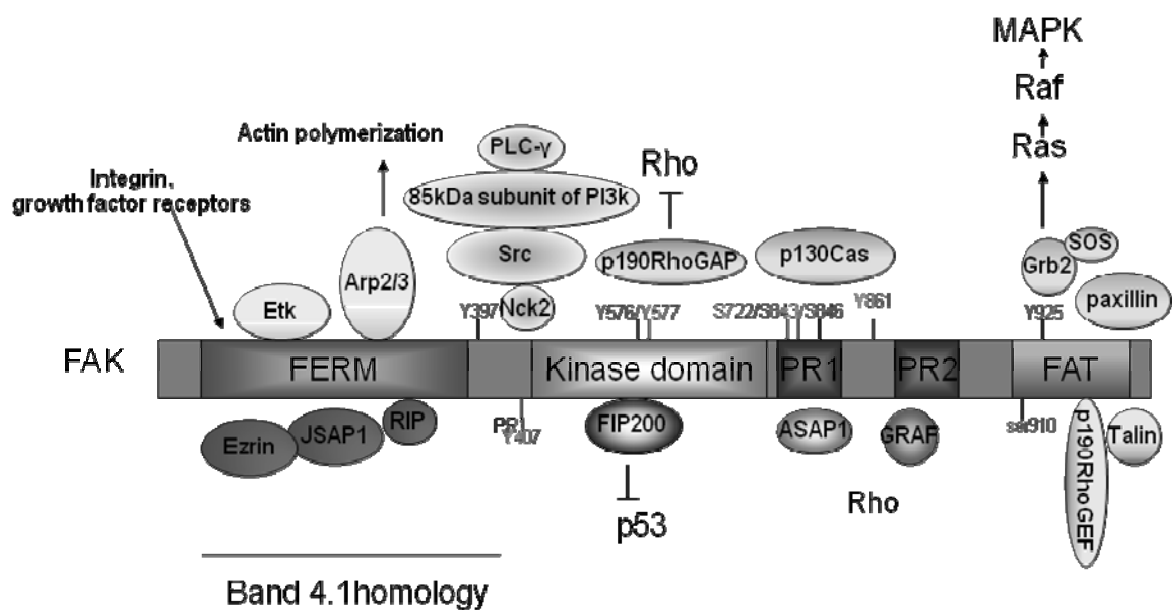


Figure 3. FAK structure, phosphorylation sites and scaffold-interaction proteins. See text for more details.

Phosphorylation of FAK

Autophosphorylation of FAK at Y397 upon the engagement of integrins with ECM recruits Src at focal adhesion sites. Local Src activation mediates further phosphorylation of FAK at Y576 and Y577, therefore a conformation change of FAK enhances the catalytic kinase activity of FAK. The Src/FAK protein tyrosine kinase complex mediates phosphorylation of other Tyr residues of FAK as well as phosphorylation of other proteins (e.g., paxillin, p130Cas) [117,118]. Phosphorylated FAK on Y861 increases the binding affinity of p130Cas to the proline-rich regions of FAK c-terminus and is crucial for sensing mechanical force [119] and H-ras induced transformation [120]. Phosphorylation of Y925 at FAT domain promotes SH2 domain-containing adaptor protein Grb2 (growth factor receptor-bound protein 2) binding to FAK, thereby allowing activation of the FAK-Grb2-Ras-MEK1-ERK2 signaling cascades. Both Y925 phosphorylation and Grb2 binding seem to be required for FAK function in promoting tumor angiogenesis [121]. Phosphorylation of Y925 not only mediates a MAPK-associated angiogenic switch during tumor progression, but also provides anti-apoptosis functions of FAK [121,122]. Although most tyrosine phosphorylation sites positively affect FAK activity, Y407 has recently been reported to negatively regulate FAK kinase activity in cell migration/invasion [123,124]. Increasing evidence indicates the importance of tyrosine phosphorylation of FAK in cell survival and migration, while the role of serine phosphorylation of FAK still remains largely unclear. Phosphorylation of FAK at Ser843 inhibits phosphorylation at Y397; Ser843 phosphorylation is increased when FAs disassemble and cells detach from the substratum [125]. The effects of phosphorylation at other serine residues, such as Ser722, Ser86 and Ser910, are still poorly understood [126,127].

Downstream effectors of FAK in cell migration and survival

FAK regulates actin cytoskeleton and cell migration by controlling focal complex assembly/disassembly at the leading edge of lamellipodia and disassembly at the rear of migrating cells [7,8]. As mentioned above, FAK phosphorylates other downstream effectors, like paxillin, p130Cas and Raf/MAPK/ERK, and triggers cell migration machinery. FAK has indirect and direct effects on Rho-family GTPases Rho, Rac, CDC42 via GEFs (guanine-nucleotide exchange factors) and GAPs (GTPase activating proteins). FAK is involved in actin and microtubule organization via effectors N-WASP and mDia [7]. In addition to the roles at FAs and cytoskeleton, FAK also has a scaffolding function in cell nuclear compartment under cellular stress conditions. A recent study [128] has shown that FAK facilitates p53 degradation and turnover via MDM2, the negative regulator of p53. This is not mediated via the FAK kinase activity but by the nuclearly-localized FERM domain. FERM F1 lobe in FAK N-terminal band 4.1 binds to p53, while FERM F2 lobe mediates nuclear accumulation and F3 lobe connects p53 and MDM2 for proteasomal degradation [114]. Interestingly, there is also a p53 binding site in the *fak* gene promoter. Overexpression of p53 negatively regulates FAK promoter activity [129,130]. This interesting connection between FAK and p53 may be of relevance to cancer progression given the defects in p53 in many types of cancers. This FAK-

dependent down regulation of p53 function seems important for the control of apoptosis [131]. The studies in our group demonstrate a role of FAK in the control of the AKT pro-survival pathway. Thus, conditional expression of a inhibitory splice variant of FAK, FAK related non-kinase (FRNK), sensitizes breast cancer cells to doxorubicin-induced apoptosis by inhibiting doxorubicin-induced AKT activation [132]. Other studies also demonstrate a relationship between FAK and AKT activation. Collagen matrix contraction-caused mechanical signal induces fibroblast cell apoptosis via disturbing β -integrin-FAK- PI3K/AKT survival signaling [133].

FAK in breast cancer development and progression

FAK regulates cell proliferation, survival and migration in cancer development and abnormal FAK expression and activity have been implicated in a panel of cancer types, including breast cancer [134-137]. Overexpression of FAK and growth factor receptors (EGFR, ErbB, c-MET) is associated with cell invasiveness, angiogenesis and poor patient survival [138-140]. Reconstitution of Tyr with Phe at different FAK Tyr residues (Y397, Y863, Y925) delays breast cancer cell migration through endothelial monolayer, indicating that the phosphorylation of FAK is crucial for breast tumor extravasation [141]. FAK also regulates tumor cell invasion and angiogenesis via supporting the expression of MMPs and VEGF [121,142]. The FAK inhibition with FRNK in our established orthotopic rat breast tumor model inhibits primary tumor formation and early phase of tumor metastasis [143]. Dual reduction of FAK and Pyk (another FAK family member) inhibits tumor formation and lung metastasis via reduced MAPK activity [144]. Mammary epithelial specific disruption of FAK in a transgenic Cre/LoxP mouse model of human breast cancer retards tumor formation and metastasis, which might be linked with the altered expression of a variety of cell cycle and metastasis-related genes [145]. This disruption of FAK also impairs mammary epithelial proliferation and the transition of premalignant hyperplasias to carcinoma and metastasis [146]. Moreover, recent data obtained with the same strategy of this breast cancer model indicates that FAK also has a role in maintaining the mammary cancer stem cell population [147].

Modulation of FAK activity in cancer progression and anticancer therapy

Given the critical role of FAK in tumor development and implication of FAK in cancer progression and treatment, modulation or disturbance of FAK signaling could be a potential target in anticancer therapy. Since *fak* complete knockout results in early embryonic lethality, FAK conditional ablation in tumor cells or animal models can be used to investigate its role in tumor progression. A few genetic knockout of FAK in specific tissue sites have been developed in animal models, such as myosin light chain 2v (MLC2v)-Cre/FAK^{fllox} [148], Cre-ER (estrogen receptor)/ FAK^{fllox} [149,150], and mouse mammary tumor virus (MMTV)-Cre/ FAK^{fllox} [145-147]. Alternatively, FAK activity can be modified by transient, constitutive or conditional expression of inhibitory splice variants of FAK, FRNK, or the FAT domain [143,151]. Overexpression of these mutant variants either induces tumor cell killing by itself, or enhances the susceptibility to cell death by various anticancer drugs *in vitro* [152,153]. For example, overexpression of

inactive FAK in breast carcinoma cells induces caspase-8 dependent apoptosis [154]. knock down approaches using shRNA or antisense RNA have also demonstrated the role of FAK in cell survival [155], and FAK inhibition with antisense RNA enhances cancer cell sensitivity to some anticancer drugs, e.g., camptothecins and docetaxel [156,157]. Recently, different FAK specific inhibitors (TAE226, PF228 and PF271) have been developed to study kinase activity and scaffold function of FAK in cell survival, proliferation and migration *in vitro* as well as tumor progression *in vivo* [158-161]. Since the above modulations of FAK *in vivo* may affect both tumor cells and stroma cells within tumor microenvironment, it has not been possible to precisely define tumor cell- specific role of FAK in anticancer drug resistance. Moreover, a more global understanding of FAK-mediated cell survival and proliferation remains unavailable. We previously showed that conditional expression of FRNK inhibited primary tumor formation and early phase of lung metastasis formation in a rat tumor and metastasis model [143]. However, the *in vivo* effect and mechanism of FAK inhibition on tumor formation and metastasis in the anticancer treatment still need to be further established. We studied the effect of conditional FRNK expression on doxorubicin sensitivity of primary breast tumors and lung metastases *in vivo* in chapter 3.

The focal adhesion scaffold protein paxillin

Paxillin structure and interactions

Paxillin is another important component of cell adhesion complex at focal adhesions. As a central scaffold protein at FA sites, paxillin contains various domains that mediate the interactions with other structural and signaling proteins (Figure 4) [162-164]. Paxillin contains five conserved leucine-rich (LD) motifs, which interact with actin-binding proteins (e.g. vinculin and actopaxin) and signaling proteins such as FAK, integrin linked kinase (ILK), and the family members of ADP ribosylation factor/GTPase activating proteins (ARF/GAPs), including G-protein-coupled receptor kinase interacting protein/paxillin kinase linker (GIT1/PKL) [165,166]. Between LD1 and LD2 there is a proline-rich motif, which binds to SH2 domain of Crk and p120 RasGAP [162]. In addition, paxillin contains four c-terminal LIM domains, which are highly conserved cysteine-rich 2-zinc-fingers-contained structures that mediate protein-protein interactions [167,168]. In paxillin, LIM2 and LIM3 domains bind to tubulin and regulate microtubule dynamics at adhesion sites, while LIM3 and LIM4 bind to protein tyrosine phosphatase (PTP)-PEST and regulate cell spreading and motility [169]. Paxillin LD2 and LD4 interact with FAK and GIT1 via its FAT domain [170,171].

Paxillin phosphorylation

Paxillin can be phosphorylated on a variety of Tyr and Ser/Thr residues [172]. Depending on the phosphorylation status, paxillin plays different roles. Non-phosphorylated paxillin is essential for fibrillar adhesion formation and fibronectin fibrillogenesis, while phosphorylated paxillin regulates the assembly of nascent adhesions and the distal part of late adhesions and induces FA turnover [173]. Phosphorylation at Tyr118 and Tyr31

regulate cell migration via paxillin-Crk complex. Phosphorylation at Ser178 is triggered by growth factor or stress response via MAPK pathway [73,174]. Phosphorylation at Ser273 regulates cell adhesion and protrusion dynamics by increasing paxillin-GIT1 binding and promoting the localization of a GIT1-PIX-PAK signaling module near the leading edge [175]. Ser188 or 190 is a target of tyrosin kinase phosphorylation and involved in cell adhesion [176]. Ser244 can be phosphorylated by CDK5 and thus reduces the interaction of FAK and paxillin in oligodendrocyte precursor cells (OPCs) [177].

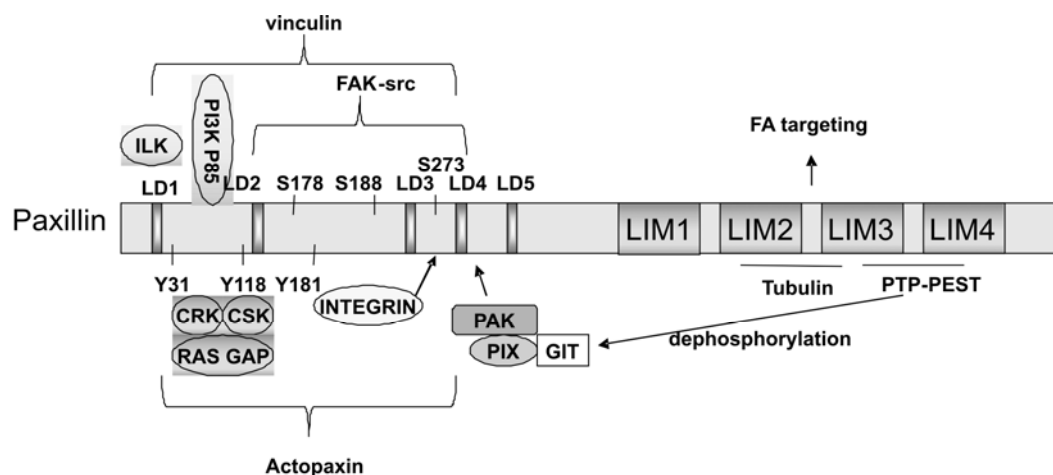


Figure 4. Adaptor protein paxillin interacts with other cytoskeleton-related proteins through LD and LIM domains. See the text for more details. Adapted from Schmalzigaug et al., 2007 [162] and Deakin et al [178]. LD domains mediate adhesion/integrin signaling and lamellipodium formation. LIM domains direct the FA targeting.

Paxillin in cell migration and survival

Assembly of paxillin LD4-PKL-PIX-Pak-Nck complex and sequential activation of paxillin (Y31/118)/Crk/p130Cas/DOCK180 cascade are two major ways to regulate cell motility via adhesion assembly and Rac activation. Explicatedly, the paxillin LD4 binds a complex of proteins containing PAK, Nck and PIX and the binding between paxillin and this complex is mediated by PKL [166,179]; PKL is a substrate of PTP-PEST which inhibits cell spreading and motility [169]. This complex is also crucial for Rac activation and cell polarization. The paxillin-Crk complex is believed to promote cell spreading and lamellipodia formation via recruitment of paxillin to focal adhesion [180]. Paxillin is also associated with the apoptotic machinery [178]. Paxillin is a substrate of caspase-3 and the cleavage of aspartic acid residues of paxillin by caspase-3 inhibits integrin-mediated cell survival signaling [181]. Recently, it is reported that Bcl-2 interacts with LD4 motif of paxillin to promote cell survival [182]. Also, paxillin shuttling through the nucleus serves as a co-activator of transcriptional factors [183], which may modulate gene expression of anti-apoptotic routes. Cell stress causes drastic changes in the cytoskeleton and focal adhesion organization including paxillin dephosphorylation [184]. However, so far it still remains largely unclear if and how paxillin regulates focal adhesion and cytoskeletal reorganization under cellular stress conditions caused by for example anticancer drug treatment. In this respect, it is important to note that FAK recruits c-Jun N-terminal kinase

(JNK) to focal adhesion sites. JNK is typically activated under cellular stress conditions (see above) and activated JNK has been observed at FAs [185]. JNK is also transiently activated after growth factor stimulation and phosphorylate paxillin at Ser178 residue [69]. Suppression of paxillin with siRNA decreases the phosphorylation level of c-Jun in skin cell transformation, indicating that paxillin may also retroact on the JNK pathway [186]. It remains elusive how JNK and paxillin interact in response to cellular stress caused by anticancer drugs, and how this relates to the growth factor-stimulated activation of JNK and cell migration.

Paxillin in cancer

Paxillin is potentially involved in several processes of tumor development. So far, most studies have focused on paxillin-related FA signaling in cytoskeleton organization, cell dynamics and survival [162,187]. Paxillin is a target of many oncoproteins, like Src, BCR/ABL [188] and E6 [189]. Moreover, it regulates gene expression via the interaction with ERK [190], Poly-A-binding protein [191], Abl and androgen receptors [192]. The deficient activation or presence of paxillin might have functions on cell migration, proliferation and survival in cancer development. However, relatively little is known about the role of paxillin in tumor development. Paxillin expression is higher in metastatic human osteosarcoma sub-cell line than less metastatic sublines and knock down of paxillin reduces cell motility. This is associated with tyrosine phosphorylation of paxillin [193]. Yet, to the best of our knowledge, no research about the role of paxillin and other alternative phosphorylation residues in breast tumor cell migration, survival or breast cancer progression has been published. In this thesis, we aim to investigate the role of paxillin, especially Ser178 residue, in cell proliferation and migration of breast cancer cell MTLn3 in tumor progression.

Aims and outline of the thesis

Understanding the molecular mechanisms of survival and migratory pathways in cancer cells is essential to better comprehending cancer progression, metastasis formation and drug resistance, thereby benefiting the development of novel anticancer treatments. The overall goal of the work in this thesis is to better understand the role and mechanism of focal adhesion-mediated signaling in the control of anticancer drug-related survival signaling of breast tumor cells *in vivo* as well as the regulation of cell migration of breast tumor cells *in vitro*. Moreover, we would like to identify the pattern of C-X-C chemokines and corresponding receptors that mediate the downstream signaling events in tumor cell migration and invasion. For these purposes we have used the well-established rat metastatic breast carcinoma cell line MTLn3 as a working model. The MTLn3 cell line is an amoeboid-like motile breast cancer cell line. The MTLn3 cell system is suitable to study anticancer drug responses, since these cells are sensitive to anticancer drugs *in vitro* [194,195]. However, they are insensitive to anticancer drugs under *in vivo* conditions [196], indicating that specific micro-environmental signaling may suppress drug toxicity.

Moreover, since this cell line demonstrates dynamic cell movement in 2D, it is also an excellent model to study growth factor and chemokine-triggered cell migration and invasion. Cytoskeleton reorganization in the context of growth factor-induced cell migration has been examined in MTLn3 cells [24, 25, 197]. Besides, previous work in our lab has established that MTLn3 cells form bigger focal adhesion under DNA-damage stress conditions [132], providing a possible link between cellular stress responses and focal adhesion organization. Furthermore, these cells can easily be used in xenograft models of breast tumor formation and experimental lung metastasis to determine the role of particular signaling pathways in metastasis formation as well as drug resistance.

We discuss the autocrine chemokine receptor CXCR3-mediated signaling pathways and biological effects in breast cancer cell in **chapter 2**. We have profiled the transcriptional expression of chemokines and receptors in MTLn3 cell line. The gene expression profiles have revealed that CXCR3 and CXCR4 are highly expressed. While the respective chemokines CXCL9, 10, 11 for CXCR3 are present, the CXCR4 ligand CXCL12 is absent. We have demonstrated that CXCR3 and the respective chemokines form an active autocrine loop and contribute to cell motility of MTLn3 cells. The MEK/ERK and PI3K/AKT pathways have been characterized as CXCR3 downstream signaling pathways that are essential for CXCR3-mediated migration. Importantly, the disturbance of CXCR3 signaling by knock down (KD) of CXCR3 with siRNA decreases cell migration to an artificial wound.

FAK has been shown to be involved in cell survival, migration and invasion. We have demonstrated that FAK is required for primary tumor formation and early stage of tumor metastasis. For this purpose MTLn3 cell lines that conditionally express the dominant-negative acting splice variant of FAK, FRNK, were used. In **chapter 3**, we show that FAK inhibition with the dominant-negative-acting mutant FRNK improves cell sensitivity to the anticancer drug doxorubicin and reduces tumor growth and outgrowth of lung metastasis, hence improving tumor sensitivity to doxorubicin. Transcriptomics analysis has revealed the differential expression of genes upon FRNK-expression. Fra-1 is prominently altered by FRNK expression as well as siRNA-mediated KD of FAK. In accordance with the finding that FRNK sensitizes MTLn3 cells to doxorubicin, downregulation of Fra-1 using siRNA approaches sensitizes the cells to doxorubicin. Interestingly, Fra-1 knock down also causes cytoskeleton reorganization and impairs cell migration associated with reduced focal adhesion turnover.

In **chapter 4**, another focal adhesion-related scaffold protein paxillin and stress activated MAPK/JNK pathway have been investigated in the context of cytoskeleton reorganization and cell cycle inhibition/apoptosis after treatment with the anticancer drug vincristine, a microtubule disrupting agent. Vincristine induces focal adhesion formation, stress fiber formation, and cell cycle arrest prior to the onset of MTLn3 cell apoptosis. Vincristine selectively activates the MAPK/JNK pathway, but not MAPK/ERK or MAPK/p38 pathway. Interestingly, we have found that vincristine treatment causes both hyper-

phosphorylation of paxillin at serine 178 residue and another unknown modification of paxillin. Phosphorylation of Ser178 paxillin and the unknown modification are dependent on JNK activity. Furthermore, SP600125, A JNK inhibitor, reduces vincristine-induced cell contractility, in association with the inhibition of F-actin stress fibers and larger focal adhesion formation. Finally, paxillin knock down reduces vincristine-induced cell contractility and focal adhesion formation. All these phenomena indicate a tight interaction between JNK and the focal adhesion-associated adaptor protein paxillin in microtubule disruption-induced cytoskeleton reorganization.

Previous researches suggest that paxillin also plays an important role in cell migration and proliferation. In **chapter 5** we further studied the potential role of JNK-mediated paxillin Ser178 phosphorylation in MTLn3 cell migration. The *in vitro* studies are performed with MTLn3 cell lines that stably express either GFP-paxillin or GFP-paxillin mutant S178A, which can not be phosphorylated by JNK. The GFP-S178A-paxillin reduces cell proliferation, focal adhesion turnover and cell migration process. Interestingly, GFP-S178A-paxillin reduces EGF-induced activation of both ERK and AKT, suggesting a potential defect in the EGFR signaling pathway. This is not observed for HGF-induced cell signaling, and GFP-S178A-paxillin is unable to block HGF-induced MTLn3 cell migration.

I briefly summarize our studies and discuss the work in related with other literature in **chapter 6**, which is followed by more discussion of further prospective work.

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CHAPTER 2

An autocrine CXCR3 activation-loop drives breast tumor cell migration and invasion through ERK and AKT signaling

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ABSTRACT

Chemokine receptor signaling has a prominent role in breast cancer cell migration in the context of metastasis formation, but the roles of many receptor-ligand pairs in this process remain unclear. Here we determined the expression profile of chemokine receptors and their respective ligands in the metastatic mammary adenocarcinoma cell line MTLn3 by microarray gene expression profiling and semi-quantitative RT-PCR. CXCR3 and its ligands CXCL10 and CXCL11 were prominently expressed in MTLn3 cells. CXCR3 activation by CXCL10 and CXCL11 induced transient intracellular Ca^{2+} mobilization. CXCL11 enhanced random cell migration and stimulated chemotaxis-driven cell invasion. CXCR3 activation by either CXCL10 or CXCL11 caused activation of both ERK and AKT. Pharmacological inhibition of MEK with U0126 or phosphoinositide-3 kinase with LY294002 inhibited the CXCL11-induced MTLn3 cell invasion. Finally, knock down of CXCR3 in MTLn3 cells inhibited cell migration into an artificial wound which was associated with reduced protrusion formation. Together, these data suggest that autocrine CXCR3 activation is important for MTLn3 tumor cell migration and invasion and a potentially important drug target to inhibit cell biological steps of breast cancer progression and metastasis.

INTRODUCTION

Malignant breast cancer is characterized by metastasis to regional lymph node, bone marrow, lung and liver. Metastasis is a complex process including detachment, migration, invasion, extravasation, homing to specific organ, and proliferation in sites of metastasis. Recent reports demonstrate that chemokines contribute to a number of tumor-related processes, such as tumor development, growth and metastasis via providing the movement direction for migrating tumor cell through chemo-attractive effect; shaping the tumor microenvironment by cell recruitment, including recruitment of leucocytes, tumor-associated macrophages and dendritic cells and angiogenesis modulation; providing survival and proliferation signaling (1-4).

Chemokines are small chemoattractant molecules with either CC or C-X-C motifs involving a wide variety of biological and pathological processes (4). Chemokines bind to their respective CC or C-X-C chemokine receptors, which belong to the subfamily of seven-transmembrane G-protein coupled receptor and are capable to activate intracellular downstream signaling (5, 6). Previous attention has been focused on the chemokine-mediated chemotaxis properties of immune cells. Recent evidence demonstrates an essential role for chemokine receptor-signaling in tumor cell survival, proliferation, chemotaxis, adhesion and migration, especially in relation to the homing of tumor cells to distant target organs (7, 8). The majority of studies on chemokine receptors in cancer progression have so far focused on CXCR4 (1, 9-11). CXCL12 (SDF-1 α) acts as a prominent chemoattractant that activates CXCR4 receptors and drives the metastasis of

various cancers to distant organs such as lung (12, 13). This is related to the activation of Ras/MEK/ERK and PI3K/AKT signaling pathways (6, 14).

CXCR3 is primarily characterized for the expression on effector and memory CD4 T cells (15), CD8 T cells and Natural Killer cells. And it contributes to the accumulation of antibody secreting cells at the sites of inflammation (16). CXCR3 has raised considerable interests in the context of cancer because of its discovery in the tumor microenvironment (17, 18). Moreover, several studies indicate the expression of CXCR3 on different cancer cells including colon cancer and melanoma (19-22). Recent data also demonstrate that overexpression of CXCR3 enhances colon cancer metastasis (22) as well as melanoma cell metastasis to lymph node (23). So far little information is available on the role of CXCR3 in breast cancer. Thus a systematic analysis of the expression of chemokine receptors and their ligands in breast tumor cells has not been performed. Besides, the role of specific chemokine-chemokine receptor pairs and possible autocrine activation mechanisms remain largely unknown. We have investigated these in the context of breast cancer cell motility and invasion.

In the present study we show the expression profile of chemokines and chemokine receptors on MTLn3 breast cancer cell. These cells express functional CXCR3 and CXCR4 as well as CXCL10 and CXCL11 but not CXCL12. CXCL11 not CXCL10 enhanced cell random migration and directional migration to collagen-coated membrane by interaction with its receptor CXCR3. We determined the role of MEK/ERK and PI3K/AKT pathway in the chemokine-induced tumor cell invasion. Finally siRNA-mediated knock down of CXCR3 inhibited MTLn3 cell migration into an artificial wound. Collectively, our data support a role of CXCR3 in breast tumor cell migration, and suggest a possible autocrine loop by which tumor cell-derived CXCR3 ligands stimulate the migration of the tumor cells.

MATERIALS AND METHODS

Chemicals and Antibodies- α -modified MEM with ribonucleosides and deoxyribonucleosides (α -MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin were from Life Technologies. Collagen (type I, rat tail) was purchased from Upstate Biotechnology and used at a working concentration of 20 μ g/ml. Propidium iodide (PI) and PI3K inhibitor LY294002 were from Sigma (St.Louis, MO). Fluo-4-acetoxymethyl (AM) ester was from Invitrogen. MEK inhibitor U0126 was from Promega Benelux B.V. Recombinant murine CXCL10, CXCL11, CXCL12 were obtained from PeproTech Inc. and used at a working concentration of 100 ng/ml. All primers for PCR were ordered from Biolegio B.V., Rabbit anti-p-ERK1/2 (p44/42 MAP Kinase, Thr202/Tyr204), rabbit anti-ERK (p44/42 MAP Kinase), rabbit anti-p-AKT (Ser473), and rabbit anti-AKT antibodies were from Cell Signaling Technology. All other chemicals were of analytic grade.

Cell Culture- MTLn3 rat mammary adenocarcinoma cells were cultured as described previously (24). GFP-MTLn3 was generated by overexpression of eGFP (enhanced GFP) followed by clone selection using G418. Selected clone had a similar behavior and morphology as parental MTLn3 cells. For experiments, cells were serum starved in α -MEM medium with 12 mM HEPES and 0.35 % (w/v) bovine serum albumin (starvation medium) for 2 hrs before experiment treatment (25). Under these conditions cell signaling was shut down as determined by cellular p-ERK levels. To study cell migration, cells were seeded on 20 μ g/ml collagen-coated transwell chambers or collagen-coated glass bottom dishes (Greiner).

Microarray Analysis- Total RNA was isolated from MTLn3 cells according to Qiagen RNeasy manufacturer's instruction. Concentration and quality of RNA were determined using lab-on-a-chip analysis. mRNA was converted to cDNA and subsequently to digoxigenin-labeled cRNA with a NanoAmpTM RT-IVT labeling kit from Applied Biosystems according to the manufacturer's instruction. Digoxigenin-labeled cRNA samples were hybridized to Rat Genome Survey Microarray (ABI) and detected with a chemiluminescent detection kit (ABI) following the manufacture's instruction. After conversion of raw signals on microarrays to expression values by Expression Array System Analyzer Software version 1.1.1, a filtering step was applied based on a signal-to-noise ratio of >3 to exclude low expressed genes.

Semi-quantitative Reverse Transcription-PCR and Real Time PCR- cDNA was synthesized from 5 μ g RNA using Superscript II Reverse Transcriptase (Invitrogen). PCR reactions were performed from 50 ng cDNA using BIOTAQ Red DNA polymerase (1U/25 μ l, GENTAUR). The sequence-specific primer pairs were separately designed by online primer design tools (<https://www.genscript.com/ssl-bin/app/primer> and http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The reactions were amplified for 35 cycles of 94°C for 30 s, 60°C for 30s, 72°C for 50s. GAPDH and β -actin were used as internal controls. PCR products were analyzed by electrophoresis on 2 % agarose gels.

Quantitative RT-PCR of CXCR3 in siRNA experiments was performed on ABI 7700 system with the SYBR Green PCR Master Mix kit (Applied Biosystem) adapted according to the manufacturer's instructions. The reactions were set up using 50 ng of template per reaction. 200, 100, 50, 25, 12.5, 6.25, 0 ng of mixture templates were run for standard curve. Reactions were performed in duplicate for 40 cycles (95°C /1min for denaturing step, 60°C /1min for annealing step and 65°C -95°C for melting curve step). Monoppeak in melting curve showed unique fragments yielded. The mRNA expression was normalized to the expression level of β -actin in each sample and relative normalized units were compared between samples.

Calcium Mobilization Assay- Cells were detached and loaded with 2 μ M Fluo-4-AM, 0.01% pluronic acid, and 1 mM probenecid in FBS-free medium for 60 min at 37 °C in

the dark. Cells were washed twice with pre-warmed FBS-free medium. Propidium iodide (1 μ M) was added to exclude dead cells after loading. Flow cytometry analysis was done on FACScalibur (BD Biosciences). First 30 seconds were recorded as baseline. Chemokine (100 ng/ml) or ionomycin (20 μ M) was added at 30 seconds. Fluo-4 and PI staining were detected at 530nm (530/30 nm dichroic bandpass filter) and 585nm (585/42nm band pass filter). At least 10⁵ cells per sample were analyzed and PI-positive cells were excluded from the Fluo-4 intensity analysis.

Random Migration Assay- 1*10⁵ GFP-MTLn3 cells were seeded in collagen-coated glass-bottom dishes and incubated overnight. After serum starvation for 2 hour, cells were visualized for 1 hour in indicated medium (starvation medium followed by addition of either 5 % FBS or 10 nM EGF or 100 ng/ml chemokine) maintained at 37°C in 5% CO₂ in a climate control unit on a Nikon Eclipse TE2000 U-inverted microscope. Migration tracks of about 50 cells were recorded framing every 5 minutes with 40x oil objective lens (Nikon), zoom 2.0 using Bio-Rad Radiance 2100 confocal system. Image acquisition was done using the LaserSharp software (Bio-Rad) with homemade auto-focus system. To determine the efficiency of stimulus, cell movement was traced for each lapse interval recorded during 1hr period and cell speed was calculated by tracking cell center in each frame. Cell surface area change between two sequent frames was calculated to represent cell dynamic and motility using ImagePro Plus (version 5.1, Media Cybernetics Inc.).

Transwell Migration Assay- Cell invasion was assayed using 24-well transwell inserts containing 6.5 mm-diameter chamber with 8 μ m-pore filter (Greiner Bio-one). Inserts were coated with collagen. After serum starvation for 1 hr, cells were trypsinized and resuspended at 5*10⁵ cells/ml in serum starvation medium. 0.1 ml of cell suspension was added to the upper chamber. 0.6 ml serum starvation medium with indicated chemoattractant was added to the lower chamber. After incubation for 8 hr, the cells on the upper surface of filters were scraped with cotton swabs to remove the non-migrating cells and non-attached cells, the filters were washed with PBS and fixed with 4.0% formaldehyde for 10 min and stained with crystal violet (0.1% w/v) for 30 min. The number of the migrating cells was counted in 3 different random areas. Data were normalized as the migration index. For the experiments with inhibitors, U0126 (10 μ M) or LY294002 (10 μ M) was added to medium in the lower chambers.

Immunoblotting- For western blot analysis, cells were starved for 4 hr and stimulated with 100 ng/ml chemokine or 10 nM EGF pre-incubated either with or without inhibitor U0126 (10 μ M) or LY294002 (10 μ M). At indicated time points, cells were washed with ice-cold PBS and TSE (10 mM Tris-HCL, 250 mM sucrose, 1 mM EGTA) and lysed in ice-cold TSE with inhibitor cocktail (1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonylfluoride, pH 7.4). After sonication, protein concentrations of cell lysates were determined using Bradford protein assay with IgG as a standard. Equal amounts of protein (25 μ g) were separated with 7.5-10% SDS-PAGE and transferred to

PVDF membrane (Millipore). Blots were blocked with 5 % w/v BSA followed by incubation with primary antibodies (1:1000) against phospho-ERK, ERK and AKT or blocked in 0.2% I-BlockTM (Applied Biosystem) followed by incubation with antibody against phospho-AKT (1:1000) and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for p-ERK, ERK, AKT or alkaline phosphatase-conjugated secondary antibody for p-AKT (1:2000). Blots were detected with ECL-Plus reagent (Amersham Bioscience) on a multilabel Typhoon imager 9400 detection system (Amersham, Bioscience) or developed in Tropix reagent (Applied Biosystem) and exposed on films.

siRNA-mediated Knock Down of CXCR3- CXCR3 knock down with SMARTpool siRNA was performed by normal transfection with DharmaFECT reagent 2 (Dharmacon RNAi Technologies). Briefly, 1.5×10^5 cells were seeded in 6-well plates overnight and transfected with 50 nM siRNA or sicontrol. mRNA was collected after 48 hr transfection. For wound healing assay, cells were detached after 24 hr post-transfection, reseeded on collagen coated glass bottom plates and incubated for another 24 hrs. The monolayer of cells was scratched to generate a wound with 200 μ l plastic tips following with overnight movies. The wound edges were visualized with 20x objective lens on Nikon Eclipse TE2000-E PFS microscope with differential interference contrast (DIC). Frames were obtained every 5 min with NIS-elements AR software (Nikon). The speed of the wound closure was calculated with ImagePro Plus software.

Statistical Analysis- Student's t-test was used to determine significant differences between two means.

RESULTS

Expression of CXC Receptors and Ligands in Metastatic Mammary Adenocarcinoma MTLn3 Cells- To study the role of chemokine receptors in cell migration and invasion, we used the highly metastatic breast cancer cell line MTLn3 as a model system. We focused on the receptors and corresponding ligands of the CXC family. First we designed primer sets for RT-PCR reactions to detect the mRNA levels of CXCR 1, 2, 3, 4 and CXCL 1, 2, 4, 5, 9, 10, 11, 12. (supplemental data S1 and S2). In addition, we performed gene expression profiling of MTLn3 cells on ABI rat full genome microarrays to evaluate the relative expression of these CXC-receptors and ligands (Table 1). In MTLn3 cells, based on both microarray analysis and RT-PCR, the expression of CXCR3 was most abundant. CXCR1 and CXCR4 were also present, but less abundant; CXCR2 was absent. For the CXC-receptor ligands, CXCL1, 2, and 5 were most abundant; they bind to CXCR2 which is absent in MTLn3 cells, excluding autocrine activation. The CXCR3 ligands CXCL9, 10 and 11 were all present in MTLn3 cells, possibly mediating an autocrine activation of CXCR3. This is not the truth for CXCR4, since no CXCL12 mRNA could be detected either on the microarray or by RT-PCR. Given the high abundance of CXCR3 as well as the corresponding ligands CXCL9, 10 and 11, we

decided to further study this interaction in MTLn3 cell migration and invasion.

Table: mRNA expression of CXC chemokines receptors and ligands in MTLn3 cells.

CXC chemokine receptors and ligands	ABI microarray ¹	RT-PCR ²
CXCR1	1.2 ± 0.4	+++
CXCR2	0.6 ± 0.1	-
CXCR3	22.0 ± 2.4	++++
CXCR4	5.6 ± 1.2	+++
CXCL1	1542.4 ± 325.0	++++
CXCL2	159.7 ± 18.4	++++
CXCL 4	1.1 ± 0.3	+
CXCL 5	62.8 ± 31.5	++++
CXCL7	0.7 ± 0.1	-
CXCL9	2.3 ± 1.5	+
CXCL10	102.5 ± 10.0	++++
CXCL11	7.1 ± 1.5	+++
CXCL12	0.5 ± 0.2	-
<i>beta-actin</i>	6570.0 ± 1546.3	+++
GAPDH	6772.8 ± 772.6	++++

¹ mRNA expression was determined by ABI microarrays. Shown are data of three independent experiments. Values shown are mean intensities of the indicated probe sets (mean ± SD, n=3).

² mRNA expression was validated using RT-PCR. Markers indicate the relative band intensities (see supplementary data S1 and S2)

CXCL10 and CXCL11 Induce Ca²⁺ Mobilization in MTLn3 Cells- Next we determined the functionality of the CXCR3 receptor. Typical chemokine receptor activation triggers the release of intracellular Ca²⁺ from intracellular free calcium stores (26). Of the three CXCR3 ligands that were present in MTLn3 cells, the affinity for CXCR3 is CXCL11>CXCL10>>CXCL9 (27). Also CXCL11 has higher potency and efficacy *in vitro* when compared to CXCL9 and 10 in activated T cells or cells transfected with CXCR3 (15). Based on the relative expression levels of these CXCR3 ligands in MTLn3 cells (see above), we decided to use CXCL10 and 11 to activate CXCR3. This effect was compared to the activation of CXCR4 by CXCL12. The calcium-ionophore ionomycin was used as a positive control. Ca²⁺ mobilization was measured by loading MTLn3 cells with Ca²⁺ indicator Fluo-4, followed by real time cell population-based analysis of the Fluo-4 intensity by flow cytometry. A clear transient increase in fluorescence intensity was observed by CXCL11 (100 ng/ml) within 1 min (Fig. 1A). Ionomycin (20 μM) caused a sustained increase in the Fluo-4 intensity. An overall slight decrease of intensity for both chemokine, ionomycin and control situation was likely due to leakage of Fluo-4 from cells. We calculated the average population intensity per 15 second time frame and plotted this as relative Fluo-4 intensity (Fig. 1B). Both CXCL10 and CXCL11 induced a transient Ca²⁺ increase which was slightly lower than that observed in the activation of CXCR4 by CXCL12 (100 ng/ml). These data demonstrate that MTLn3 cells contain functional CXCR3 and CXCR4 receptors.

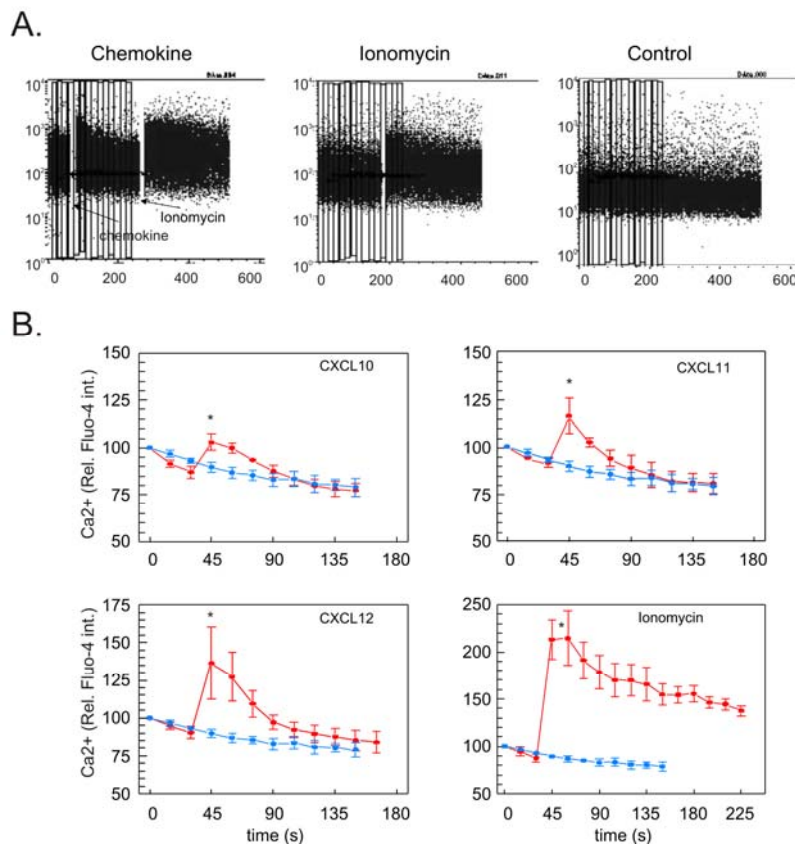


Figure 1: Chemokine receptors CXCR3 and CXCR4 are functional in MTLn3 cells. Intracellular free Ca^{2+} was determined by Fluo-4 fluorescence intensity analysis using flow cytometry as described in Materials and Methods section. **A.** Representative dotplots for Fluo-4 intensity vs. time (sec) are shown the treatments with CXCL11 (100 ng/ml), ionomycin (20 μM) and control condition respectively. **B.** Relative average Fluo-4 intensities of the cell population were calculated for 15 sec time intervals after stimulation with CXCL10, CXCL11 and CXCL12 (100 ng/ml, each) or ionomycin (red line) and control conditions (green line). Data shown are results from 3 independent experiments (mean \pm SD; $n=3$).

CXCR3 Activation Causes MTLn3 Cell Migration- Activation of chemokine receptors can induce actin cytoskeletal rearrangement thereby facilitating cell morphology and motility. To investigate the effect of CXCR3 ligands on the behavior of MTLn3 cells, we performed live cell imaging of GFP-labeled MTLn3 cells to study the migration behavior of the cells. Cells were serum starved for 4 hrs followed by addition of either CXCL10 or CXCL11. Again we included CXCL12 for comparison, but also growth factor EGF as well as normal fetal bovine serum (FBS). Cell motility was visualized for 1 hr, followed by cell tracking analysis to calculate both cell speed and cell dynamics. CXCL11 induced a clear increase in the random MTLn3 cell migration, which was comparable to that observed with EGF (Fig. 2A and C). CXCL10 also stimulated random cell migration. Despite the fact that CXCL12 induced a drastic Ca^{2+} mobilization (see above), it did not induce random cell migration (Fig. 2C). Since relative absence of cell speed does not necessarily mean that cells remain fully static, we also analyzed the relative movement of cells based on their relocation between two time points which was calculated as the percentage of average cell surface change to total cell surface area (see Fig. 2B). Apparently, control cells are dynamic (i.e. 20 % surface change between time points; Fig. 2B), but hardly migrate away (Fig. 2A). In contrast, cells exposed to either CXCL11 or EGF were more dynamic (i.e. ~ 30 % average surface change; Fig. 2D) and also had longer migration trajectories (Fig. 2A).

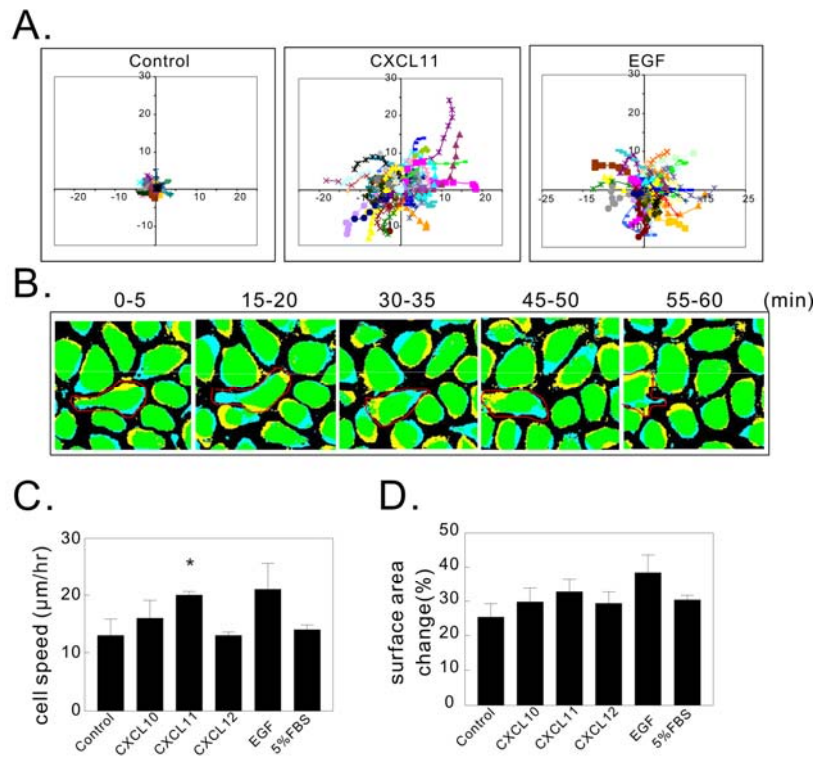


Figure 2: CXCR3 activation induces increased random cell migration. GFP-MTLn3 cells were serum starved for 2 hrs followed by live cell imaging of cell migration in the absence (control) or presence of the indicated chemokines (100 ng/ml), EGF (10 nM) or 5 % FBS. **A.** Images were analyzed by ImagePro-Plus software to determine individual cell tracks, or **B.** Relative dynamics between different time points; green indicates the overlapping area between two sequential frames, yellow indicates the cell area in previous frame and blue in the present frame. **C.** Average cell speed was calculated per hour. **D.** The ratio of surface area change

to total area was calculated as the average over the 1 hr period. Results shown are from at least 3 independent experiments (mean \pm SD; n=3).

Realizing that chemokines presented on the endothelial cell surface typically trigger integrin affinity and mediate leukocyte arrest on endothelial wall, we presumed that chemokines in tumor cells would also enhance integrin-mediated adherence. Therefore, we also examined the capability of CXCR3 ligands to promote cell adhesion and spreading on collagen. We discriminated cell phenotypes as adherent (but still rounded), half-spread, fully spreading and elongated in shape. CXCL11, and to a lesser extent CXCL10, stimulated the spreading of MTLn3 cells on collagen (Fig. 3A). Cells treated with EGF and FBS attached and spread even faster. Interestingly, under these conditions both EGF and FBS stimulated the rapid formation of focal adhesions as determined by staining for phosphorylated-Tyr118-paxillin. This was not observed for CXCL10, CXCL11 or CXCL12 (data not shown) indicating differential downstream signaling from CXCR3 and CXCR4 compared to EGFR.

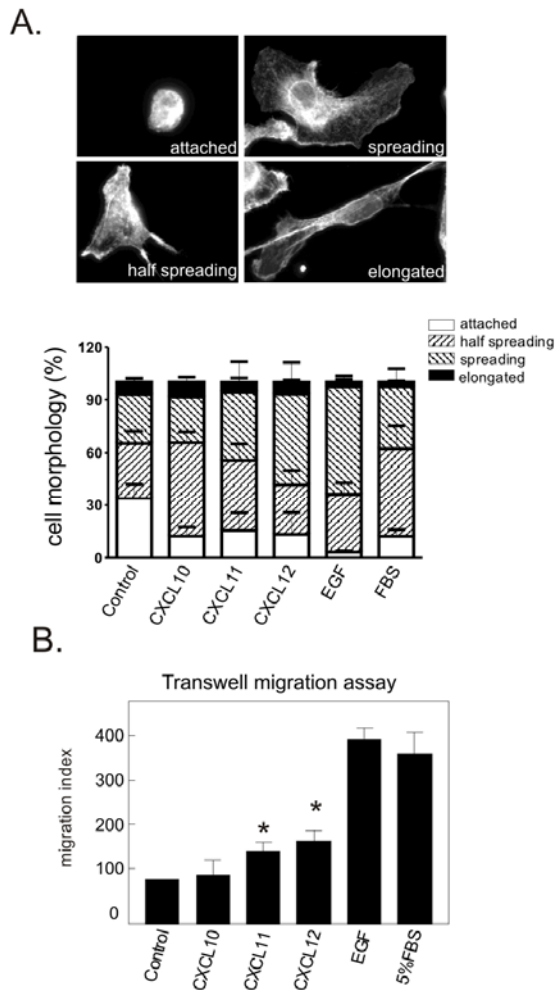


Figure 3: CXCL11 and CXCL12 facilitate cell adhesion and directional migration.

Serum starved MTLn3 cells were detached and reseeded on collagen coated coverslips in starvation medium in the absence or presence of chemokines (100 ng/ml), EGF (10 nM) or 5 % FBS. Cells were fixed and stained with Hoechst, pY118-paxillin and F-actin. **A.** Cell phenotype was classified as attached (left top), half spreading (left bottom), fully spreading (right top) or elongated (right bottom). **B.** To determine directional invasion, 50,000 serum starved cells were seeded on collagen-coated membrane with 8 μ m pore diameter and were attracted to lower chamber containing indicated chemoattractant at similar concentration as in panel A. After 8 hrs the relative numbers of invading cells were quantified as indicated in Materials and Methods section. Results shown are from 3 independent experiments (mean \pm SD; n=3).

Migration and invasion into extracellular surroundings are major features of the metastatic capability of tumor cells. This is typically stimulated by chemotactic activities of growth factors and chemokines. To investigate the role of CXCR3 and CXCR4 in this process, we evaluated the ability of serum-starved MTLn3 cells to invade through collagen coated microporous membranes towards a concentration gradient of either CXCL10 or CXCL11; again both CXCL12 and EGF were used for comparison. CXCL11 and CXCL12 facilitated cell directional migration by $64 \pm 21\%$ and $87 \pm 22\%$ compared to control (starvation medium only), while CXCL10 was not effective at all. Both EGF and FBS showed stronger potential ($315 \pm 24\%$ and $283 \pm 49\%$ respectively; Fig 3B). The combined data indicate that the activation of CXCR3 can induce cell adhesion, migration and invasion in metastatic MTLn3 cells.

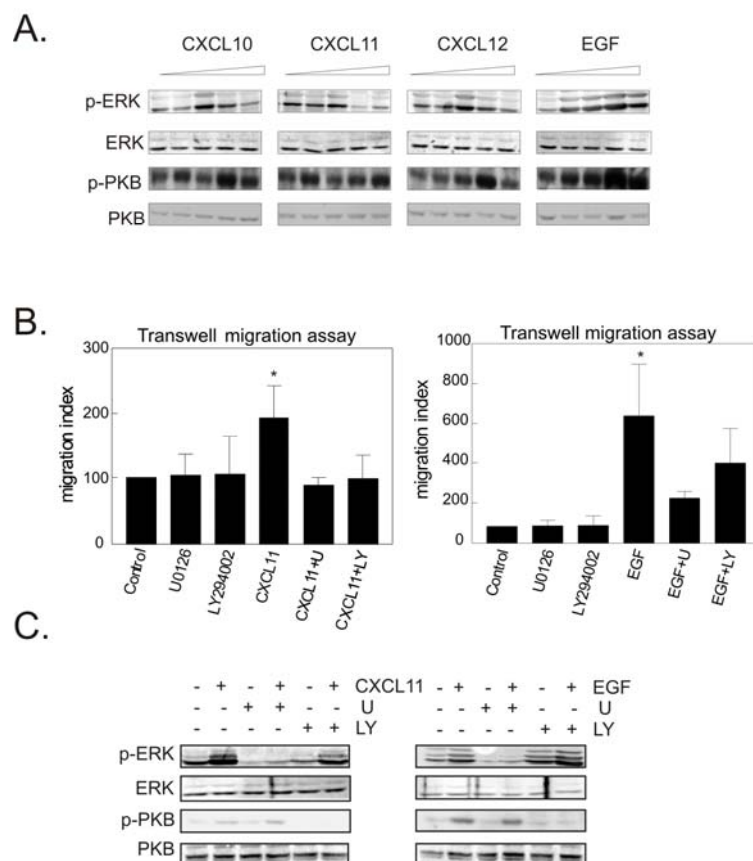


Figure 4: Chemokines trigger cell directional migration via ERK and AKT pathways. A.

Serum starved MTLn3 cells in 6 cm dishes were exposed to CXCL10, CXCL11, CXCL12 and EGF for 0, 5, 10, 20 or 60 mins followed by sample collection for western blotting. Blots were stained for p-ERK, ERK, p-AKT and AKT. **B.** To determine the role of the MEK/ERK and PI3K/AKT pathway in MTLn3 cell directional migration, cells were exposed to either CXCL11 or EGF; U0126 and LY294002 were added to bottom chambers prior to the addition of cells in the chambers. **C.** The effects of U0126 and LY294002 on ERK and AKT activation by CXCL11 and EGF were determined by western blotting similarly as in panel A. Results shown are from 3 independent experiments (mean \pm SD).

Activation of ERK and AKT Pathways Drives CXCL11-Induced Cell Migration- Next we investigated the signaling pathways that mediate CXCL11-induced cell migration. Several signaling pathways are downstream of chemokine-induced receptor activation, including the phosphoinositide-3 kinase (PI3K)/AKT pathway and the Ras/Raf/ERK pathway (28). Therefore, we first examined the activation of these pathways in more detail. Serum-starved MTLn3 cells were exposed to CXCL10, CXCL11, CXCL12 or EGF for various time periods, followed by the analysis of ERK and AKT activation by western blotting. CXCL11 and CXCL12 caused a transient and strong activation of ERK and AKT after 10 min of exposure, which reduced thereafter again. The activation of ERK and AKT by CXCL10 was less strong, while EGF induced a more sustained activation of ERK and AKT which lasted up till 60 min (Fig. 4A). Next we determined whether these pathways were involved in MTLn3 cell migration. For this purpose we used U0126, a selective inhibitor for MEK1/2, upstream of ERK, and LY294002, which inhibits PI3K, upstream of AKT. The transwell migration assay was performed to determine the role of both pathways in the directional migration/invasion. Both U0126 and LY294002 inhibited MTLn3 cell migration through the transwell membranes induced by CXCL11 and EGF (Fig. 4B). Importantly, both U0126 and LY294002 also inhibited the activation of ERK and AKT induced by either CXCL11 or EGF (Fig. 4C). These data indicate a role for both ERK and AKT activity in CXCL11-induced cell migration of MTLn3 cells.

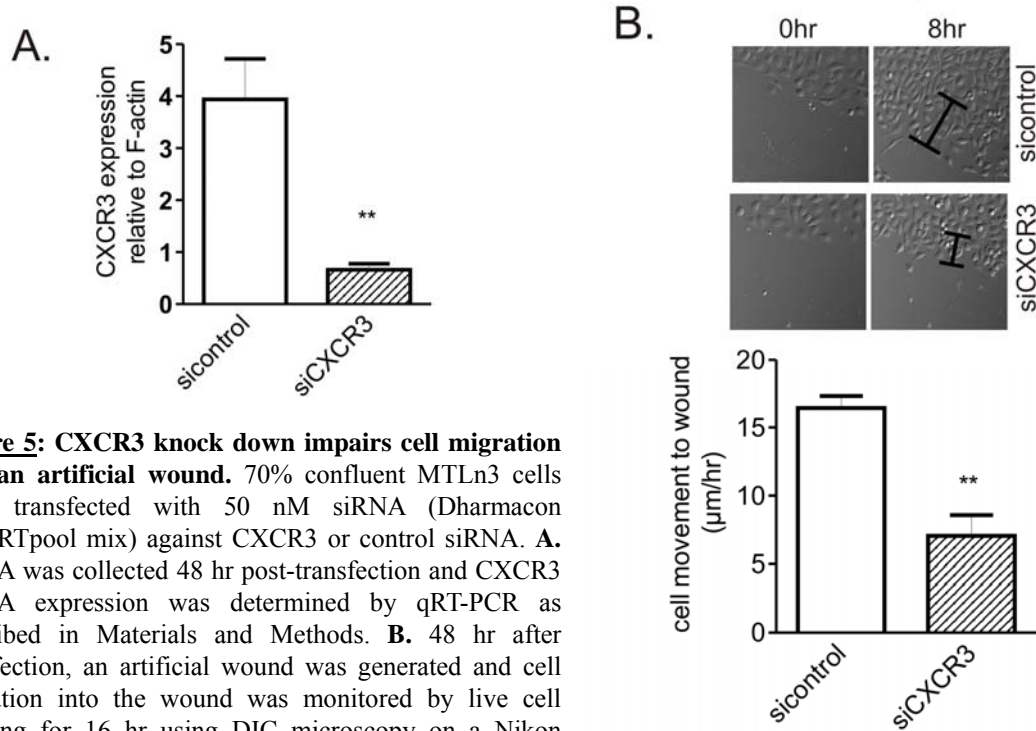


Figure 5: CXCR3 knock down impairs cell migration into an artificial wound. 70% confluent MTLn3 cells were transfected with 50 nM siRNA (Dharmacon SMARTpool mix) against CXCR3 or control siRNA. **A.** mRNA was collected 48 hr post-transfection and CXCR3 mRNA expression was determined by qRT-PCR as described in Materials and Methods. **B.** 48 hr after transfection, an artificial wound was generated and cell migration into the wound was monitored by live cell imaging for 16 hr using DIC microscopy on a Nikon TE2000-E PFS microscope. Top panels are individual frames from representative movies (see also supplemental movie M1 and M2 for control siRNA and siCXCR3, respectively). Cell velocity was defined as wound edge migration per hour (μm/hr). Data shown are results from 3 independent experiments (mean ± SD; n=3).

CXCR3 is Essential for MTLn3 Cell Migration- The above data indicate that exogenous CXCL11 can drive MTLn3 cell migration. Given the fact that MTLn3 cells express CXCR3 as well as CXCL9, CXCL10 and CXCL11, we suggest that a possible autocrine loop is essential in normal cell migration. To further investigate it, we modulated the expression of CXCR3 in MTLn3 cells using siRNA-mediated knock down (KD) with Dharmacon SMARTpool siRNA mixes. Non-targeting siRNA SMARTpool mixes were used as control. 48 hrs after transfection, CXCR3 mRNA expression was reduced by more than 80% as determined by qRT-PCR (Fig. 5A), without affecting cell viability. Next we performed live cell imaging of the wound healing assay using differential interference contrast (DIC) microscopy. This allowed us not only to determine the efficiency of cell migration, but also to monitor the behavior of individual cells (see supplemental movies M1 and M2). CXCR3 KD MTLn3 cells were less effective in closing the artificial wound and had a slower velocity of 7.1 μm/hr compared to 16.4 μm/hr for sicontrol cells (Fig. 5B). Interestingly, while sicontrol cells were highly motile and showed large protrusions when entering the wound, siCXCR3 cells were more static and did not show much protrusion formation. Nevertheless, mitotic events were observed under both conditions indicating that CXCR3 KD does not affect cell viability (see supplemental movies M1 and M2). These data suggest that CXCR3 is essential for cell migration of MTLn3 cells, most likely through autocrine signaling by either CXCL10 or CXCL11.

DISCUSSION

In the present manuscript we investigated the expression of CXC-receptors and ligands in the highly metastatic breast cancer cells MTLn3 and determined the role of the prime CXC-receptors in tumor cell migration and invasion. Our data indicate that, firstly MTLn3 breast tumor cells have abundant expression of CXCR3 in association with expression of corresponding ligands CXCL10 and CXCL11. Secondly, the CXCR3 receptor in MTLn3 is functional and responds strongly to CXCL11 thereby inducing activation of Ca^{2+} mobilization as well as activation of ERK and AKT. Thirdly, the activation of CXCR3 drives cell migration and invasion which is dependent on Ras/MEK/ERK and PI3K/AKT signaling pathways. MTLn3 cell migration is dependent on CXCR3 since knock down of CXCR3 prevents spontaneous cell migration into a wound which is associated with reduced cell protrusion formation. Together, these data are suggestive of an autocrine CXCR3 activation loop in MTLn3 cells, whereby expression of CXCL10 and CXCL11 induces cell migration and invasion processes. These data suggest that targeting CXCR3 in breast cancer can be a suitable way to inhibit local tumor cell invasive properties thereby preventing intravasation and tumor cell dissemination.

Chemokines have the ability to activate second messenger G- protein, the downstream activation of phospholipase $\text{C}\beta$ (PLC β), PI3K/AKT and Ras/MEK/ERK pathways as well as c-Src-related non-receptor tyrosine kinases (29). Indeed, activation of CXCR3 receptors in MTLn3 cells activates Ca^{2+} mobilization, which is most likely mediated by PLC activation, as well as activation of the PI3/AKT and Ras/MEK/ERK pathways. CXCL12 can also induce the phosphorylation of the focal adhesion-associated kinase FAK at Y397 and Y577 and other focal adhesion-associated adapter proteins such as paxillin and Crk (13). These phosphorylation events are likely to modulate the focal adhesion dynamics in migrating cells by CXCL12. However, we did not observe a significant change in the phosphorylation of pY397-FAK and pY118-paxillin after either CXCL11 or CXCL12 treatment (data not shown). Chemotaxis and invasion of tumor cells involve not only cytoskeleton reorganization but also the secretion of various hydrolytic enzymes like matrix metalloproteinase (MMPs). Using gelatin zymography, we detected the activity of MMP2 and MMP9 in MTLn3 serum-free conditioned culture medium but did not observe a significant increase in their activity after chemokine addition (data not shown).

So far there are three studies by Fulton and co-workers on the role of CXCR3 in breast cancer progression (18, 30, 31). High CXCR3 expression was associated with poorer overall survival in a clinical study in 75 women diagnosed with early-stage breast cancer. The inhibition of CXCR3 by the AMG487 compound or shRNA gene silencing reduced spontaneous lung metastasis formation (18, 30). Intriguingly, overexpression of the CXCR3 ligand CXCL9 in the same cell line reduced primary tumor growth and almost fully inhibited lung metastasis formation. This was associated with enhanced T-cell and

Natural Killer cell infiltration in tumor tissue (31). Apparently, there is a tight balance between the autocrine activation of tumor cell CXCR3 by secreted CXCR3 ligands and the chemoattractant activity of these ligands to attract immune cells. Too much secretion of CXCR3 ligands may activate the immune response, and inhibiting this secretion may not be beneficial to ultimate disease outcome. So far it remains unclear whether CXCL10 and CXCL11 overexpression have a similar effect as CXCL9 and whether this effect is also observed in other breast tumor models. Moreover, it is unclear at which expression level of CXCR3 ligands, the breast tumor biology will tip from autocrine activation of tumor cells to promote migration and invasion towards enhanced T-cell infiltration to compromise tumor growth. In colon cancer and melanoma, high CXCR3 expression is associated with reduced patient survival (32, 33), while overexpression of CXCR3 promotes spontaneous metastases of colon carcinoma cells to lymph nodes (32) and knock down of CXCR3 with antisense reduces metastasis to lymph node of B16F10 melanoma cells (23). Further studies are required to determine the role of CXCR3 in breast cancer metastasis formation to lymph node, lung and bone, and whether autocrine activation is an essential component of the metastasis formation. Moreover, the relative importance of CXCL9, CXCL10 or CXCL11 expression in the metastatic process of breast tumor cells as well as other tumor cells including colon and melanoma, needs to be determined. Thus, increased levels of these ligands in the primary tumor will promote the migratory behavior of tumor cells or promote tumor cell killing by T- and NK cells. Alternatively, high levels of these CXCR3 ligands in the dissemination target organs, including lymph node, lung, bone and liver, could be crucial to facilitate homing and induce adhesion, migration and invasion of the tumor cells, ultimately resulting in local metastasis formation.

In this study we have compared the potentials of CXCR3 activation by CXCL11 with CXCR4 activation by CXCL12 and EGFR activation by EGF. CXCR4 activation, similar to CXCR3, was effective in inducing Ca^{2+} mobilization, ERK and AKT activation and chemoattractant-induced invasion. However, spontaneous cell migration was less affected. CXCL12 was not expressed by MTLn3 cells, excluding possible autocrine activation in the primary tumor. Indeed, accumulating evidence indicates that local high level of CXCL12 drives metastasis formation of breast tumor cells to lung, which is dependent on the functionality of CXCR4 receptors. Thus, knock down of CXCR4 with siRNA (10, 11) or pharmacological inhibition of CXCR4 impaired breast tumor cell invasion and delayed the formation and metastases of breast cancer (1). Given the combined expression of CXCR3 and CXCR4 in our breast cancer cells, we could anticipate that these receptors synergistically affect tumor metastasis formation by autocrine activation of CXCR3 in the local primary tumor microenvironment and by activation of both CXCR3 and CXCR4 at distant organs such as lung. Indeed in colon cancer, both CXCR3 and CXCR4 positive primary tumors have a worse disease progression (22). Therefore, pharmacological intervention of both CXCR3 and CXCR4 may be beneficial in anticancer treatment regimens. Here it is noteworthy that the effects of CXCR3 and CXCR4 activation in MTLn3 cells were in all respects not as strong as observed from EGFR stimulation. Also,

in vivo the EGFR signaling is essential for metastasis formation of MTLn3 cells (34). Therefore, on the long term the intervention of both CXCR3 and/or CXCR4 together with EGFR antagonists may be most effective in preventing metastasis formation.

In conclusion, our current data indicate an important role of CXCR3 in controlling cell migration and invasion properties of breast tumor cells. Future work will focus on the role of CXCR3 as well as the individual CXCR3 ligands in breast tumor cell biology *in vivo*.

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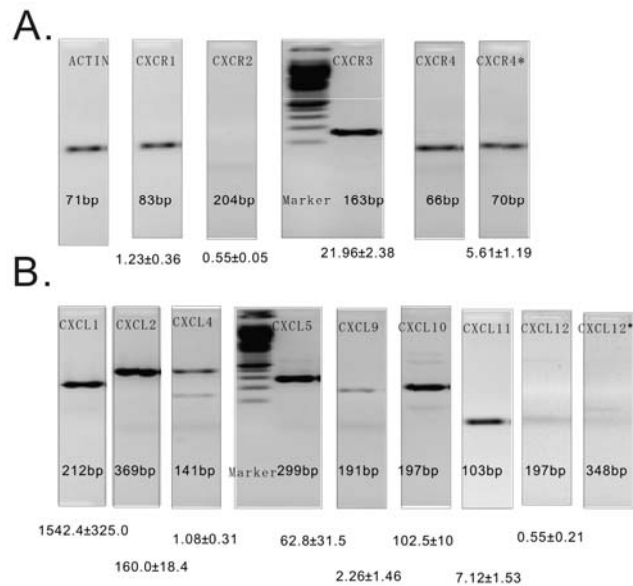
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Supplemental data S1: The primer sets used in RT-PCR for mRNA expression of chemokines and chemokine receptors.

Name	Genebank No.	Forward primer	Reverse primer	product
CXCR1	U71089	5' CTGTCTCTGCCTTTTGCCA 3'	5' GCTTCACCCAGGACCTCAT 3'	83bp
CXCR2	U70988	5' ATAGTGTGTTCCCTTGCAATA 3'	5' AACTGTCAATAT CTCCACTG 3'	204bp
CXCR3	NM_053415	5' TCTATGCCTTTGTGGGAGTGAA 3'	5' CTGAATTACAAGCCCAAGTAGGAG 3'	163bp
CXCR4	U90610	5' CAATGGGTGGTAATCCTGGTC 3'	5' CGGTACTTGTCTGTCATGCTCC 3'	66bp
CXCR4*	U90610	5' CGCCCTCCTCCTGACTATCC 3'	5' ACAGATGTACCTGCCGTCCC 3'	70bp
CXCL1	NM_030845	5' TAAACCAGCTCCAGCACTCC 3'	5' GCGGCATCACCTTCAAACCTCT 3'	212bp
CXCL2	NM_053647	5' GGAAGCCTGGATCGTACCTG 3'	5' CCCTCTGACTGCGTCTGTTT 3'	369bp
CXCL4	NM_001007729	5' GCTGCTTCTTCTGGGTCTGC 3'	5' AGGCTGGTGATGCGTTTGAG 3'	141bp
CXCL5	U90448	5' TGGCATTCTGCTGCTGTTC 3'	5' AAGTGCATTCCGCTTTGTTT 3'	299bp
CXCL9	NM_145672	5' ATTCCTCATGGGCATCATCTT 3'	5' TCTCCGTTCTTCAGTGTAGCG 3'	191kb
CXCL10	BC058444	5' AGCCAACCTTCCAGAAGCAC 3'	5' TGCGGACAGGATAGACTTGC 3'	197bp
CXCL11	NM_182952	5' GCCCTGCAAACATTCTACGC 3'	5' TCTTTAGCCCTTTAGACTGCC 3'	103bp
CXCL12	BC078737	5' CCCTGCCGATTCTTTGAGA 3'	5' CCTTTGGGCTGTTGTGCTT 3'	197bp
CXCL12*	BC078737	5' CACCTCGGTGTCTCTTGCT 3'	5' CCTTTGGGCTGTTGTGCTTA 3'	348bp
GAPDH	NM_017008	5' GTGAGGTGACCGCATCTTCTT 3'	5' CGTGGGTAGAGTCATACTGGAAC 3'	205 bp
β -actin	NM_031144	5' CAGCTTCTCTTTAATGTCACGCA 3'	5' TGACCGAGCGTGGCTACA 3'	71bp

Supplemental data S2: PCR products of CXC chemokines and chemokine receptors. The numbers below gels are raw data from microarray.



Supplemental movies M1 and M2: 70% confluent MTLn3 cells were transfected with 50 nM siRNA (Dharmacon SMARTpool mix) against CXCR3 or control siRNA. 48 hrs after transfection, an artificial wound was generated and cell migration into the wound was monitored by live cell migration imaging for 16 hrs using DIC microscopy on a Nikon TE2000-E PFS microscope. Movie M1 is a representative movie for control siRNA KD MTLn3 cells and movie M2 is a representative movie for siCXCR3 KD cells.

CHAPTER 3

Role of Fos related antigen-1 (Fra-1) in focal adhesion kinase (FAK) mediated chemoresistance of mammary adenocarcinoma cells

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Running title: Focal adhesion kinase and breast cancer drug resistance.

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ABSTRACT

Focal adhesion kinase (FAK) is essential for tumor cell survival, migration and metastasis formation. The exact role and mechanism of FAK in chemoresistance of solid and metastatic tumors remain unclear. Conditional expression of focal adhesion kinase-related non-kinase (FRNK) in established MTLn3 mammary fat pad tumors prior to doxorubicin treatment reduced tumor progression by more than eighty percent. Moreover, in experimental metastasis model doxorubicin treatment combined with FRNK significantly reduced lung metastasis outgrowth by almost seventy percent without affecting metastasis size. Neither FRNK nor doxorubicin alone affected either fat pad tumor or lung metastasis growth compared to control conditions. Genome-wide gene expression profiling identified Fra-1, an activator protein (AP)-1 family member, as an essential mediator in this process. Loss of FAK suppressed Fra-1 expression, while loss of Fra-1 reduced cell adhesion and migration in association with increased stable focal adhesion formation. Finally, Fra-1 knock down sensitized MTLn3 cells towards doxorubicin, whereas Fra-1 overexpression suppressed cell death when FAK was absent. A model is proposed whereby FAK-mediated signaling is linked to Fra-1 expression and cell survival thereby mediating drug resistance.

INTRODUCTION

Chemoresistance of distant tumor metastases is a major problem in the treatment of cancer. This is mainly due to increased and unsuppressed proliferation and survival signaling in cancer cells. Increased expression of the non-receptor tyrosine kinase focal adhesion kinase (FAK) is implicated in various tumors, including breast tumors (1, 2). FAK expression is positively associated with breast cancer development and poor disease prognosis (3, 4). The accumulative data indicate a critical role of FAK in tumor cell migration, invasion, proliferation and survival processes enhancing the metastatic capacity of tumor cells and possibly resulting in a resistant phenotype (5-7).

Once cells adhere to ECM, tyrosine phosphorylation at multiple residue sites of FAK and consequent protein interaction signaling result in activation of survival signaling pathway (8, 9). Inhibition of FAK by siRNA or expression of FAK deletion mutants, including FRNK or FAT, induces the onset of apoptosis itself and/or sensitization to anticancer drug treatment in a variety of cancer cell types *in vitro* including breast cancer cells (10-12). Furthermore, systemic RNA interference-based FAK knock down inhibits ovarian tumor growth and sensitivity towards docetaxel and cisplatin (13-16). However, this has not been investigated in breast cancer. Given the non-directed targeting of the siRNAs, it remains unclear whether this is related to direct effects on tumor, stromal or vascular endothelial cells. So far a direct relationship between specific inhibition of FAK function in tumor cells *in vivo* and sensitization towards anticancer drugs needs to be established. Besides, it remains unclear whether such a role of FAK would be similar in primary tumors as well as in distant metastases.

Fra-1 (Fos-like antigen 1, also named Fosl-1), is a component of activator protein (AP)-1 transcription factor. The AP-1 transcription factor complex comprises of Fos, Jun and ATF family members (17). It plays an important role in regulating tumor cell proliferation, survival, migration and invasion through modulation of genes expression, for example cyclin dependent kinases, cyclins, metalloproteinases and adhesion molecules. Recent studies show that Fra-1 expression is associated with breast cancer (18-20) regarding lower or no expression in ER-positive luminal-like breast cancer cells and higher expression in migratory mesenchymal-like breast cancer cells (19, 20). Overexpression of Fra-1 in epithelial-like breast cancer cells promotes cell proliferation (19). Moreover, Fra-1 is crucial for the migratory and invasive behavior of different cancer cells (19, 21). This in colon carcinoma cells is related to the suppression of beta1-integrin activation, thereby preventing the initiation of RhoA/ROCK-dependent contractility and focal adhesion stabilization (21). In addition, Fra-1 expression is linked to the control of cell survival in different cell types (22, 23). So far the relationship between FAK and Fra-1 signaling in susceptibility towards anticancer drugs remains unclear.

To investigate sensitization towards anticancer drugs as a consequence of selective tumor cell-related FAK inhibition in the *in vivo* situation, conditional inhibition of FAK in tumor cells is required. The rat mammary adenocarcinoma MTLn3 cell line is a good model to study the role of FAK in drug sensitivity, since MTLn3 cells are susceptible towards a range of anticancer reagents *in vitro* including cisplatin, etoposide and doxorubicin (24, 25), but resistant *in vivo* ((26) and the present work). Importantly, we have recently established a conditional MTLn3 stable cell line, MTLn3-tetFRNK cells which allows to conditionally express FRNK in a doxycyclin-dependent manner *in vivo* (7). This strategy can conditionally modulate FAK function at any given moment without affecting the initial tumor formation and metastasis formation.

Our data indicate for the first time that conditional expression of FRNK in metastatic MTLn3 breast tumor cells sensitizes both primary tumors in the mammary fat pad and experimental lung metastases towards doxorubicin treatment. Gene expression profiling of MTLn3-tetFRNK cells indentified Fra-1 as a target in FAK-mediated signaling. While RNAi-mediated FAK knock down suppressed Fra-1 expression, knock down of Fra-1 did not affect FAK expression but sensitized cells to doxorubicin-induced apoptosis. Moreover, overexpression of Fra-1 inhibited the sensitization to doxorubicin when FAK was suppressed. Our data support a model whereby FAK signaling drives Fra-1 expression and modulates the sensitivity towards anticancer drugs.

EXPERIMENTAL PROCEDURES

Chemicals and antibodies- Alpha modified minimal essential medium without ribonucleosides and deoxyribonucleosides (α -MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin, Lipofectamine-Plus and geneticin (G418 sulphate) were from Life Technologies. Doxorubicin (doxo) and doxycycline were from

Sigma. Hoechst 33258 and the Alexa-488 protein labeling kit were from Molecular Probes. Hygromycin was from Roche. Primary antibodies were anti-HA (clone 3F10) and anti-HA (clone 12CA5) (Roche), anti-FAK (Upstate, Lake Placid, NY), rabbit anti-Fra-1 antibody (Santa Cruz Biotechnology), mouse anti-myc (Roche), rabbit anti-p-ERK (Cell Signalling), mouse anti-paxillin (Transduction), rabbit anti-paxillin-PY118 and anti-FAK-PY397 (Biosource). All other chemicals were of analytic grade.

Cell culture, stable cell lines and transient knock down- MTLn3 rat mammary adenocarcinoma cells and MTLn3-tetFRNK cells were cultured as previously described (7). To establish retroviral transduced stable cell lines, the construct p-BABE-puro-Fra-1 tagged with 8x myc (27) was used. p-BABE-puro empty vector was used as a control. MTLn3 cells were infected by retrovirus in the presence of polybrene (10 µg/ml) overnight. Stable transfectants were obtained by selection in 2 µg/ml puromycin for 1 week. Cells were transfected with smartpool Dharmacon siRNA mixes against FAK or Fra-1 (50 nM) using Dharmafect reagent 2. siRNA against GFP was a negative control. All experiments were performed 48-72 hr post-transfection.

In vitro doxorubicin exposure and cytotoxicity assays- For cell death analysis, after 24 hr of pre-incubation with doxycycline to express HA-FRNK, cells were exposed to 2 µM doxorubicin in α -MEM for 8hrs. Cell death was determined by staining the pooled attached and detached cells for Annexin-V-Alexa488/Propidium Iodide (AV/PI) as previously described (24). For apoptosis analysis, cells were exposed to 2 µM doxorubicin (or DMSO as a control) for 1hr in Hanks' balanced salt solution/HEPES, followed by recovery of the cells in α -MEM containing 2.5% (v/v) FBS for an additional 7 hrs. Apoptosis was determined with cell cycle analysis as previously described (28) on FACS-Calibur (BD Biosciences) and expressed as the percentage of sub (G₀) positive cells. For soft agar colony growth assays, MTLn3-tetFRNK cells were cultured for 24 hrs in the absence or presence of doxycycline and exposed for 1 hr in Hank's/HEPES buffer with different concentrations of doxorubicin (0, 0.01, 0.05, 0.1, 0.5, 1 and 5 µM). Cells were recovered in α -MEM (2.5% (v/v) FBS). After 24 hr, 12,500 viable cells (resuspended in 1 mL α -MEM containing 0.33% (w/v) agar, 5% (v/v) FBS and PSA) were plated on top of a bottom agar layer (2.5 mL of α -MEM containing 0.66 % agar, 5% (v/v) FBS and PSA). After two weeks cells were stained with MTT and the number of colonies was quantitated with Image J as previously described (29).

In vivo tumor growth, metastasis formation and doxorubicin treatment- Primary tumors and experimental metastases were induced as described previously (5). Briefly, 1×10^5 viable cells in 0.2 mL PBS were injected into the lateral tail vein or 1×10^6 cells in 0.5 mL PBS were injected into the fat pad of female Fischer 344 rats. Nine days after injection, doxycycline (400 mg/mL in 2.5% (w/v) sucrose) was added to the drinking water; control animals received 1.5 % (w/v) sucrose in their drinking water which resulted in equal drinking volumes. Three days later animals were treated either with 6 mg/kg doxorubicin or with PBS (intraperitoneal injection). After 33 (primary tumor) or 28 days

(experimental lung metastases), animals were anesthetized with pentobarbital and the primary tumor or the lungs were excised. The weight was determined and tumors and lungs were fixated (5). After ink injection in the lungs, the number of lung surface metastases was counted. Lung metastasis size was determined by classification of the metastases size in HE-stained lung sections in five groups: ranging from 1 (small) to 5 (large).

Gene expression profiling- Cells were pretreated with doxycycline for 24 hr to induce FRNK expression. Total RNA was isolated with the Qiagen RNeasy mini kit and digested with RNase-free DNase (Sigma). Eluted RNA was controlled by lab-on-a-chip analysis. mRNA was converted to cDNA and subsequently to digoxigenin-labeled cRNA with a NanoAmpTM RT-IVT labeling kit (Applied Biosystems). Digoxigenin-labeled cRNA samples were hybridized to the microarrays (Applied Biosystems Rat Genome Survey Microarrays) and detected with a chemiluminescent detection kit (Applied Biosystems). After conversion of the raw signals of the microarrays to expression values by Expression Array System Analyzer Software Version 1.1.1, a filtering step based on a signal-to-noise ratio of >3 was applied. Out of all 26,848 genes on the array, 13,206 (49.1%) passed this step and were regarded as expressed in the MTLn3 cells. The probe-to-gene annotation release version 12_05 was used for gene annotation. Subsequently, a median-normalization step was performed by computing a gene-by-gene difference between individual array and the reference array (the array whose overall log-intensity is the median of all array overall log-intensities), and subtracting the median difference from the log-intensities on that array, so that the gene-by-gene difference between the normalized array and the reference array is 0. BRB-array software tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) were applied to identify genes that were differentially expressed among classes by using a multivariate permutation test and random variance F-statistics. Global-test gene ontology (GO) analyses with GoMiner tool (<http://discover.nci.nih.gov/gominer/>) were carried out to analysis group genes of which the expressions were differentially regulated among different treatments.

Quantitative RT-PCR- cDNA was synthesized from RNA using oligo(dT)₁₂₋₁₈ primers and Superscript reverse transcriptase (Invitrogen Life Technologies). The sequence-specific primer pairs were separately designed by online primer design tools (<https://www.genscript.com/ssl-bin/app/primer> and http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and ordered from Biolegio (Nijmegen). Primer sets were: Fra-1(NM_012953), left: 5'-AGAGCTGCAGAAGCAGAAGG-3', right: 5'-CAAGTACGGGTCCTGGAGAA-3', length: 182bp. JunB (NM_021836), left: 5'-ATCACGACGACTCATACGCA-3', right: 5'-CGATAAGGATCTGCCAGGTT-3', length: 248bp. β -actin ((NM-031144), left: 5'-CAGCTTCTCTTTAATGTCACGCA -3', right: 5'-TGACCGAGCGTGGCTACA- 3', length: 71bp) was used as internal standard. Quantitative RT-PCR was performed on ABI7700 system with the SYBR Green PCR Master Mix kit (Applied Biosystem).

Fluorescence recovery after photobleaching (FRAP) and random migration- To determine cytoplasmic mobility of GFP-paxillin, a 1.8 μm -wide strip spanning approximately the width of the cytoplasm (without any focal adhesion) was photobleached by a short bleach pulse (100 ms) at 100% laser intensity (120 to 160 μW ; argon laser at 488 nm). Fluorescence recovery within the strip was monitored using 100-ms intervals and low laser intensity (450 to 750 nW) to avoid photobleaching by the probe beam. Approximately 10 cells were averaged to generate one FRAP curve in a single experiment. To determine the turn over of individual focal adhesions, photobleaching was applied to a small area covering a single focal adhesion for 1 s with laser intensity of 50 μW . Redistribution of fluorescence was monitored with 100 ms time intervals at 7.5 μW starting directly after the bleach pulse. Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve in a single experiment. All measurements were performed at 37 °C using a heating stage with temperature control and the experiments were performed on at least three different days. Images were analyzed with Image software Zeiss. The relative fluorescence intensity of individual focal adhesion was calculated at each time interval as follows: $I_{\text{rel}}(t) = (F A_t / F A_0)$, where $F A_t$ is the intensity of the focal adhesion at time point t after bleaching, $F A_0$ is the average intensity of the focal adhesion before bleaching. The fluorescent curves were analyzed with non-linear regression analysis with GraphPad Prism 5 (34).

For live cell random migration, GFP-MTLn3 cells were knocked down with siRNA against Fra-1 for 24 hrs and seeded on collagen-coated glass bottom plates. Live cell imaging were captured on the second day in a climate control chamber. Cell random migration were recorded on a Nikon TIRF microscope system (Eclipse TE2000-E, Nikon with automated stage) with framing every 5 minutes for 4 hrs using NIS-elements AR software (Nikon).

Immunoblotting and immunofluorescence- Doxorubicin-exposed cells or frozen lung/tumor tissue were prepared and separated by 7.5% SDS-PAGE and transferred to PVDF membranes (Millipore) as described before (5). Blots were blocked and probed with primary antibody (overnight, 4°C) followed by incubation with secondary HRP-coupled antibody and visualized with ECLplus reagent (Amersham Biosciences, Uppsala, Sweden) by scanning on a Typhoon imager 9400 (Amersham Biosciences). Immunostaining of tissue sections and cells was done as described before (5) and visualized using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60X Nikon objective.

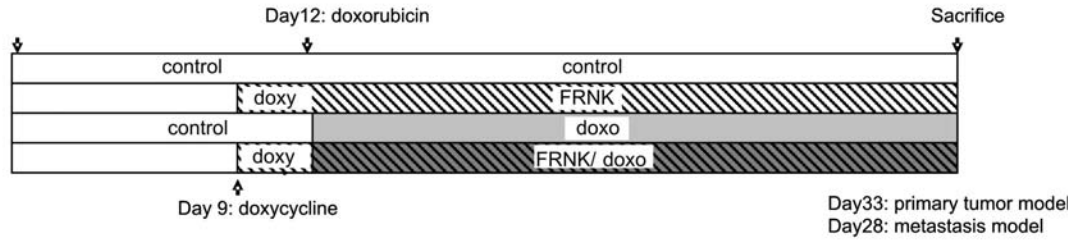
Statistical analysis- Student's t test was used to determine significant differences between two means ($p < 0.05$).

RESULTS

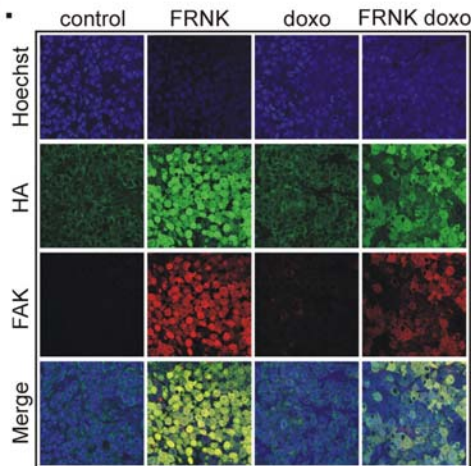
HA-FRNK Expression Sensitizes MTLn3 Breast Tumors to Doxorubicin. To test whether inhibition of FAK sensitizes MTLn3 cells towards doxorubicin treatment under *in vivo* conditions, two models were used: a primary tumor model, in which the tumor cells were injected into the fat pad of the rats and an experimental metastasis model, in which the cells were injected into the lateral tail vein of the rats. To modulate FAK function, we used our previously characterized MTLn3-tetFRNK cell line that conditionally expresses the HA-tagged dominant negative acting splice variant of FAK, focal adhesion kinase-related non-kinase (FRNK). In both experimental set-ups, HA-FRNK in the tumor cells *in vivo* was induced after nine days by addition of doxycycline to the drinking water until the end of the experiment (Fig. 1A and 1B). Three days later animals were treated either with saline or a sub-lethal dose of doxorubicin (6 mg/kg) (see for experimental setup Fig.1A). While all animals survived after the doxorubicin treatment, doxorubicin caused a temporal and small loss of weight (data not shown) indicative of medium sub-lethal toxicity. Expression of HA-FRNK starting at day nine till the end of the experiment did not affect the growth rate of the tumor (Fig. 1C). Also, treatment with 6 mg/kg doxorubicin at day twelve alone was not effective in reduction of tumor growth. In contrast, exposure to doxorubicin accompanied by the expression of HA-FRNK caused a significant decrease of tumor growth. In agreement with tumor volumes, neither HA-FRNK nor doxorubicin exposure alone altered the total tumor weight. In contrast, the combination of HA-FRNK and doxorubicin strikingly prevented tumor growth (Fig. 1D). This indicates that FAK-dependent signaling mediates a resistant phenotype against doxorubicin treatment.

A.

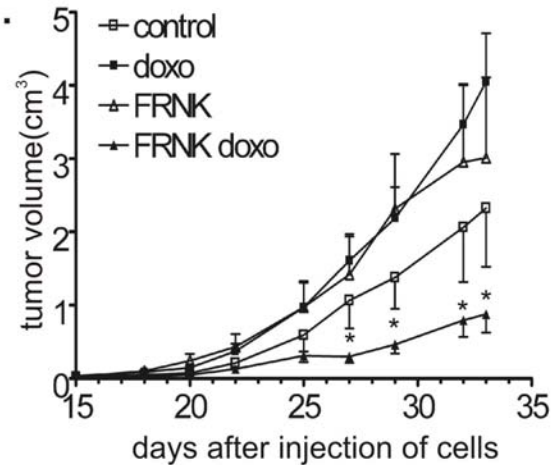
Day 0: 1×10^6 cells fat pad (primary tumor model)
 1×10^5 cells tail vein (metastasis model)



B.



C.



D.

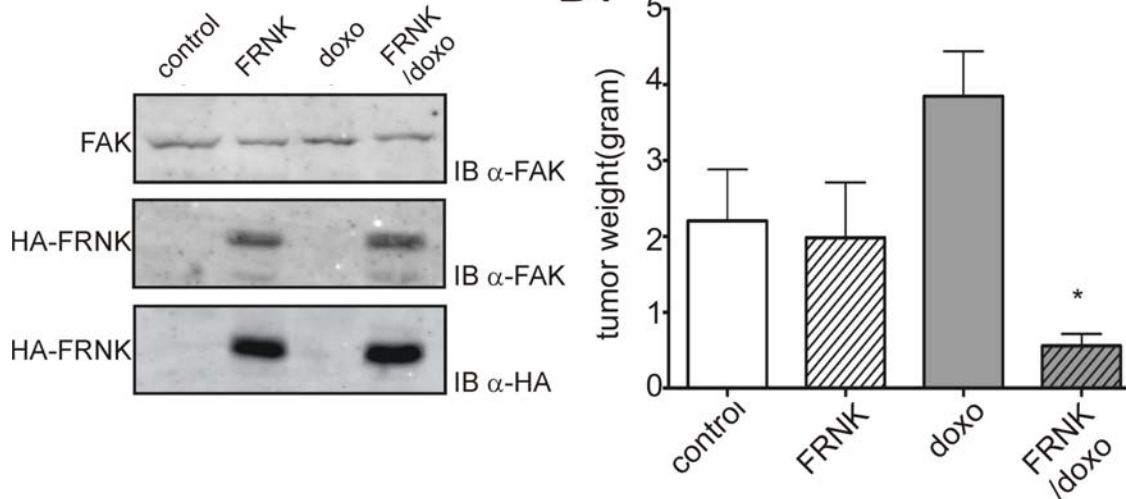


Figure 1: HA-FRNK alleviates doxorubicin resistance of primary MTLn3 tumors. In vivo experimental set-ups for primary tumor and experimental metastatic models as described in experimental procedures (A). Primary tumors were isolated, fixated, sectioned and expression of HA-FRNK was determined by fluorescence microscopy and immunoblotting stained with antibodies against C-terminal FAK and HA. Hoechst staining showed cell populations (B). During the primary tumor experiment, tumor growth was followed by measuring tumor size as described in experimental procedures (C). The weight of the primary tumors was determined (D).

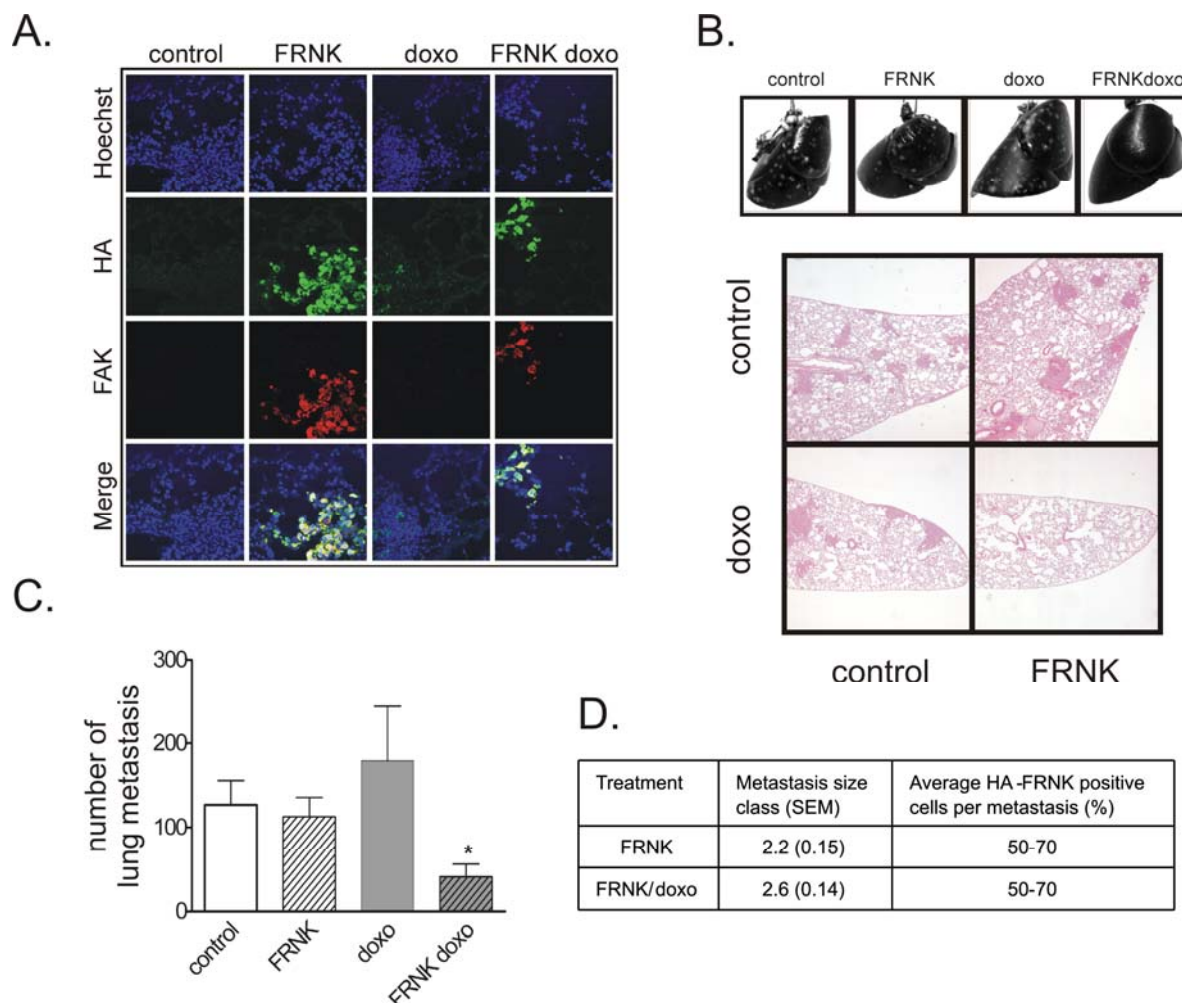


Figure 2: HA-FRNK sensitizes lung metastases to doxorubicin treatment. Animals were injected with MTLn3-tetFRNK cells and after 9 days rats were exposed to doxycycline or left untreated followed by a single treatment with doxorubicin on day 12. At day 28, animals were sacrificed and lungs were evaluated for HA-FRNK expression (A). Lung metastases formation was evaluated in a macro level on ink injected lungs (top panel) and microscopical level in HE-stained lung tissue sections (bottom panel) (B). The number of surface lung metastasis was quantified (C). Size of the individual remaining metastases and the percentage of HA-FRNK positive cells in these metastases were determined in lung sections stained for HA-FRNK of animals from the FRNK and FRNK/DOXO groups (D).

HA-FRNK Sensitizes Experimental Lung Metastases Towards Doxorubicin Treatment.

Chemotherapy is the first line treatment for metastatic breast cancer. The difference in tissue microenvironment between lung metastases and primary tumor may have an alternative effect on survival signaling programs and drug resistance. Therefore, we also investigated whether FAK is involved in resistance of MTLn3 lung metastasis. For this purpose MTLn3-tetFRNK cells were injected in the tail vein, and after nine days when micro-metastases were formed, animals were treated with doxycycline and challenged with doxorubicin treatment on day twelve (6 mg/kg). The total number of lung metastasis was evaluated at the end of the experiment. Doxycycline treatment resulted in conditional expression of HA-FRNK in the majority of lung metastases (Fig. 2A). Neither inhibition

of FAK nor doxorubicin exposure alone reduced the number of lung metastases. In contrast, expression of HA-FRNK followed by doxorubicin treatment three days later did induce a dramatic reduction of the lung tumor burden (Fig. 2B and C). The number of surface metastases was in agreement with the overall lung tumor burden as determined by histopathology (Fig. 2B). Despite the fact that HA-FRNK expression caused a decrease in the number of lung metastases by more than 70 % (Fig. 2C), the size of the remaining metastases, irrespective of HA-FRNK expression, was minimally reduced. Hardly any remaining micro-metastases were observed in any of the experimental conditions. Importantly, around 90 % of these remaining metastases contained HA-FRNK positive cells, accounting for an average of about 50-70% of all cells (Fig. 2D). These data indicate that the combined effect of HA-FRNK expression and doxorubicin treatment eliminated large numbers of micro-metastases, but did not affect further outgrowth of the remaining metastatic lesions.

FAK Signaling Regulates Fra-1 Expression. Next, we performed a systematic analysis of HA-FRNK-associated signaling that could explain the sensitization to doxorubicin. Thus, genome-wide expression profiling was carried out in MTLn3-tetFRNK cells; MTLn3 tet-on cells were used as a control to exclude potential effects by doxycycline. Out of 13,206 genes expressed in the MTLn3 cells, 494 annotated genes ($p < 0.05$, $FC > 1.5$ or < 0.67) were differentially expressed upon HA-FRNK expression (individual genes are listed in Supplemental Table 2). Since false discovery rate (FDR) for all genes was larger than 0.05, we also performed gene ontology (GO) pathway analysis to define alternatively affected biological and molecular pathways and gene sets. 23 GO-groups were identified to be differentially expressed (Table 1). Fra-1 and JunB, both activator protein-1 (AP-1) family members, were identified as prominently down regulated genes after HA-FRNK expression and parts of the GO-group *regulation of transcription* (Table 1 and Fig. 3A, Supplemental Table 2); other AP-1 family members such as c-Jun and c-Fos were not affected by HA-FRNK expression (supplemental Fig. 1). Verification of Fra-1 and JunB expression by qRT-PCR showed a 37 % decrease of Fra-1 by HA-FRNK expression compared to control (Fig. 3B). No significant decrease was observed for JunB. To further validate the effect of HA-FRNK, we performed a FAK knock down with Dharmacon Smartpool siRNA. FAK knock down in MTLn3 cells significantly reduced Fra-1 mRNA expression by more than 50% as determined by qRT-PCR (Fig. 3C). This was in agreement with a reduction of Fra-1 protein levels to around 50 %. Fra-1 knock down did not affect the levels of FAK expression (Fig. 3D).

Table 1: FRNK-induced differential alteration in gene ontologies as determined GO-miner.

Gene Ontology	p value ¹ down	genes down	p value up	genes up
epidermal cell differentiation			0.01	KA17
B cell differentiation	0.04	CLC, TPD52		
cell cycle	0.03	CDC25B, CDK2, HRASLS3, MAPRE3		
mitosis	0	CDC25B, CDK2, MAPRE3		
regulation of transcription, mitotic	0	FOSL1, JUNB		
regulation of cell growth	0.02	WISP1		
negative regulation of cell growth	0.05	NPPB, OKL38		
cell motility			0.05	TPBG
regulation of cell shape	0.05	GNA12, MYH9		
GPI anchor biosynthesis	0.01	GPAA1, PIGM		
protein myristoylation	0.03	NMT2		
protein modification	0	GGCX, PPT2		
protein amino acid acylation	0.07	PPARGC1A		
protein sumoylation			0.02	PIAS3
ubiquitin cycle	0.03	RNF40, STAMBP, UBE2D3		
receptor recycling	0.05	RAB40C		
respiratory chain complex IV			0.02	COX6C
mitochondrial outer membrane	0.04	AKAP1, HK2		
endoplasmic reticulum	0.01	CYP3A13, DDOST, NPL4, NUCB2, TPD53		
endoplasmic reticulum membrane	0	PIGM		
integral to endoplasmic reticulum membrane	0	DHRS9, ELOVL6, SLC35D1		
GPI-anchor transamidase complexes	0.05	GPAA1		
nucleus	0.02	ACTN4, ANKRD1, ANXA7, CDK2, DCK, ETV4, FOSL1 , HAVCR1, HNRPR, KPTN, MAF, MEF2D, NPL4, NR1H2, NUCB2, PHLDA1, PIM1, PKD1, POU2F2, PPARGC1A, PRKACA, RNF40, STAMBP, TCFE2A, TLE4, TNPO2, WDR3		

¹P < 0.05, P-value is either up or down in individual gene ontologies

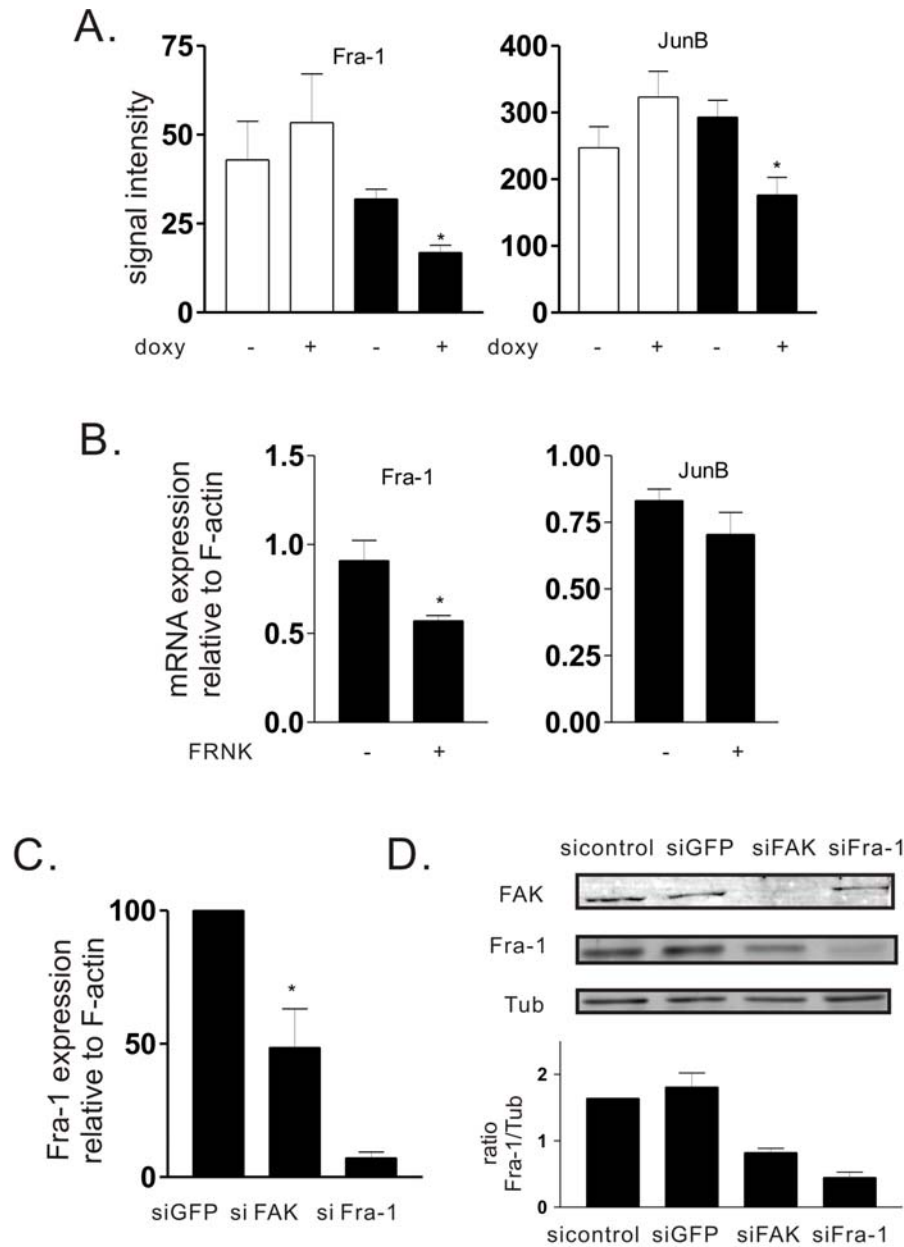
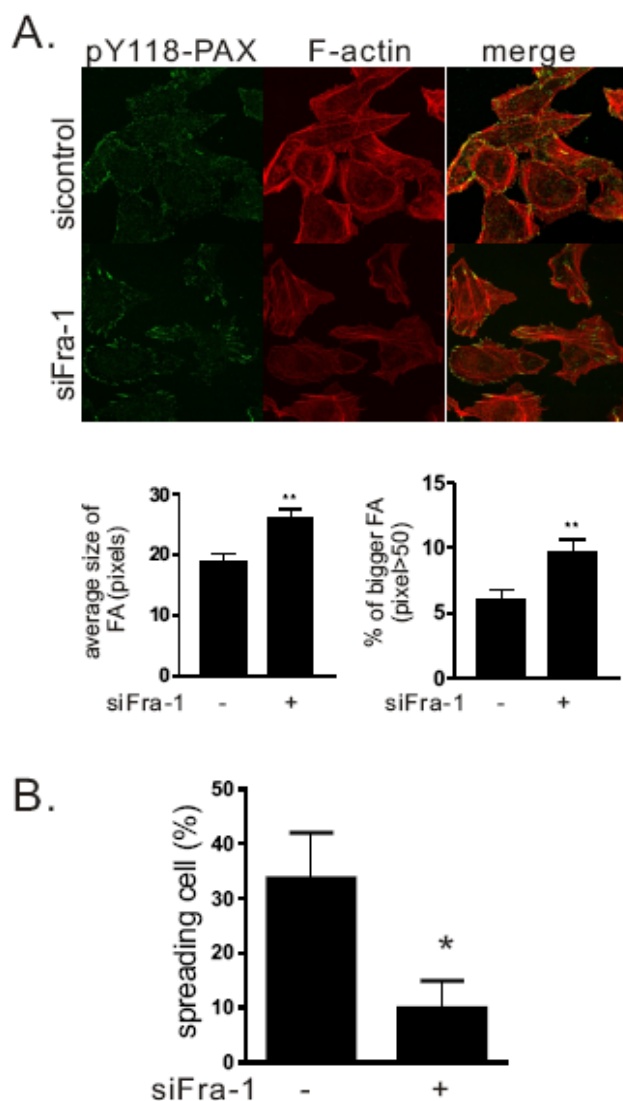


Figure 3: Fra-1 expression is dependent on FAK signaling. Microarray profiling was performed and analyzed as mentioned in experimental procedures. Shown are the expression levels of *fra-1* and *junB* in MTLn3-tet-on (open bars) and MTLn3-tetFRNK cells (closed bars) after O/N treatment with or without doxycycline (A). *fra-1* and *junB* levels were determined by qRT-PCR in MTLn3-tetFRNK cells with or without O/N treatment with doxycycline (n=3, mean \pm SEM; asterisk indicates P=0.017) (B). After transfection of normal MTLn3 cells with siFAK and siFra1, *fra-1* levels were determined by qRT-PCR (n=3; mean \pm SEM; P = 0.016) (C) and protein levels of Fra-1 and FAK were determined by immunoblotting; Fra-1 expression levels were quantified by densitometry (D).

Fra-1 Provides Cell Survival Against Doxorubicin-Induced Apoptosis. Fra-1 has been implicated in cancer progression, cell survival and cell migration. Therefore, next we studied the relationship between FAK and Fra-1 in the context of focal adhesion organization and control of apoptosis. Intriguingly, while Fra-1 knock down in MTLn3

cells did not affect FAK expression, immunofluorescence with phosphorylated paxillin indicated the presence of larger focal adhesions and increased actin filaments after Fra-1 knock down (Fig. 4A). This was associated with delayed cell spreading on collagen when the cells were replated (Fig. 4B). Moreover, FRAP experiments with GFP-paxillin MTLn3 cells demonstrated a stabilization of focal adhesion turnover after Fra-1 knock down (Fig. 4C). Thus, the diffusion of GFP-paxillin in the cytoplasm shows no difference in Fra-1 knock down compared to control condition (The reduced mobile fraction (R_f) in control condition is 0.8851 and half-life τ is 0.5498s and R_f in Fra-1 knock down is 0.8891 and τ is 0.5015s). The turnover rate of GFP-paxillin at FAs significantly decreased when Fra-1 was knocked down (R_f in control condition is 0.7560 and τ is 1.239s and R_f in Fra-1 knock down is 0.7385 and τ is 1.846s). This effect of Fra-1 knock down on focal adhesion dynamics was linked with reduced cell motility, as determined by random cell migration of MTLn3 cells (Fig. 4D). Together, these data indicate that Fra-1 activity directly links to both focal adhesion dynamics, size and cell migration properties. This may affect focal adhesion derived signaling.



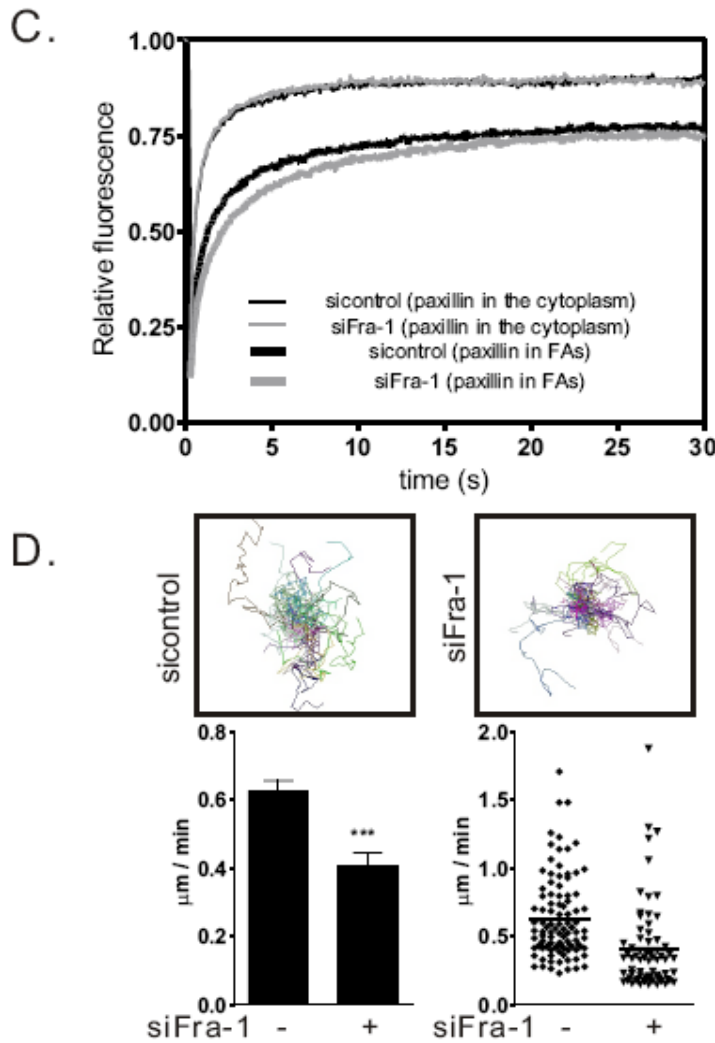


Figure 4: Fra-1 KD affects focal adhesion organization, dynamic and cell migration. MTLn3 cells were treated with siFra-1 or siGFP for 48hrs and cells were fixed and stained for pY118-paxillin and F-actin. Following image acquisition by CLSM, the average size of focal adhesion (FA) as well as the percentage of large FAs (# pixels > 50) was determined with ImagePro Plus software (A). siFra-1 knocked down cells were detached and re-seeded on collagen-coated coverslips for 4 hrs and cell morphology were observed with phase contrast light microscope and cell attachment were determined by the percentage of spreading cells (B). GFP-MTLn3 cells were treated with siFra-1 or sicontrol (siGFP) followed by FRAP analysis on a Zeiss confocal microscope as described in experimental procedures. Shown are representative FRAP curves of GFP-paxillin in the cytosol and at focal adhesions (C). For random cell migration, Fra-1 was knocked down as described above in GFP-MTLn3 cells. Random cell migration was

performed 24 hrs after plating and analyzed as described in experimental procedures. Data shown are representative individual cell movements for three independent experiments. The top panels indicate the cell tracks of individual cells in sicontrol (left) and siFra-1 (right) conditions (D).

Next we determined the sensitivity of MTLn3 cells towards doxorubicin when both FAK and Fra-1 were affected. Conditional expression of HA-FRNK sensitized MTLn3 cells towards doxorubicin-induced apoptosis (Fig. 5A). Since loss of FAK function decreases Fra-1 expression (Fig 3C and 3D), we hypothesized that loss of Fra-1 would also sensitize cells to doxorubicin-induced apoptosis. Similar to HA-FRNK expression, Fra-1 knock down indeed rendered cells more susceptible to doxorubicin (Fig. 5B). We anticipated that increased expression of Fra-1 would protect cells against loss of FAK. Therefore we generated a stable MTLn3 cell line with increased Fra-1 expression with retroviral vector pBABE-Fra-1 (Fig. 5C). Importantly, although Fra-1 overexpression did not affect FAK and paxillin levels in MTLn3 cells, it inhibited the onset of apoptosis under conditions when cells were depleted from FAK by siRNA pretreatment (Fig. 5D). All together these data indicate a role of FAK in controlling Fra-1 expression, while Fra-1 expression is essential for cytoprotection against doxorubicin-induced cell killing in breast tumor cells.

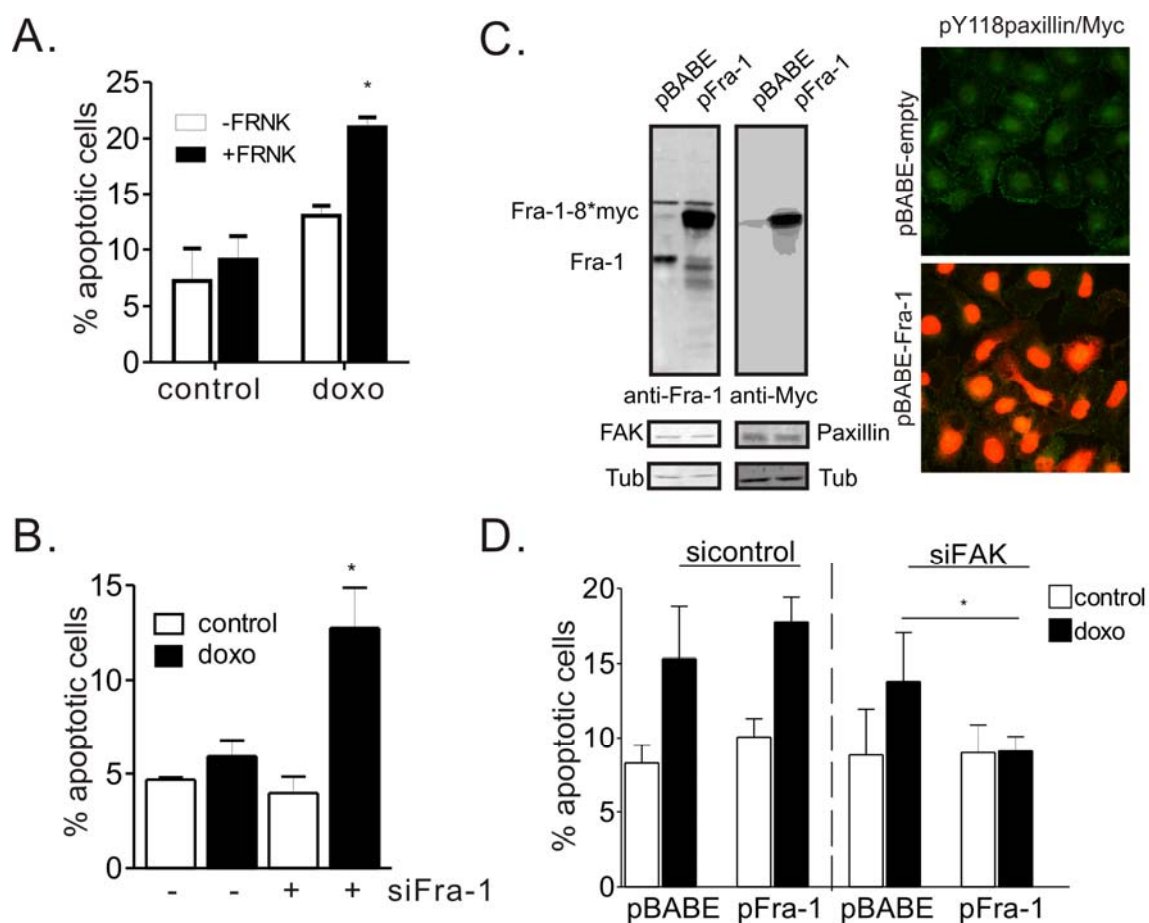


Figure 5: Fra-1 determines sensitivity towards doxorubicin-induced apoptosis. MTLn3-tetFRNK cells were treated overnight with doxycycline to induce HA-FRNK expression and then exposed to doxorubicin (2 μ M) for 8 hrs. Cell apoptosis was determined by Annexin/PI staining and flow cytometric analysis (A) (n=3; mean \pm SEM; asterisk indicates $P < 0.05$). MTLn3 cells were treated with siFra-1 or sicontrol for 48hrs and exposed with doxorubicin (2 μ M) for 8 hrs and followed with subsequent apoptosis analysis (B). (n=3; mean \pm SEM; asterisk indicates $P < 0.05$). MTLn3 cell lines expressing Myc-tagged Fra-1 were generated as described and characterized for Myc-Fra-1 expression using western blotting and immunofluorescence; Myc-Fra-1-MTLn3 cells were also examined for FAK and paxillin expression (C). MTLn3-pFra-1 and MTLn3-pBabe control cells were treated with siFAK or sicontrol and followed by doxorubicin treatment (2 μ M) for 8 hrs and apoptosis was determined as in B (n=3; mean \pm SEM; asterisk indicates $P < 0.05$) (D).

DISCUSSION

Using an orthotopic breast tumor model and an experimental lung metastasis model in combination with conditional doxycyclin-dependent expression of a FAK deletion mutant, FRNK, we investigated the role and mechanism of FAK signaling in the regulation of chemosensitivity of breast tumor cells towards doxorubicin. We demonstrate that 1) tumor cell specific inhibition of FAK in both primary tumor and lung metastases re-sensitizes breast tumor cells towards doxorubicin; 2) induction of FRNK expression selectively affects the expression of a panel of target genes, of which the AP-1 transcription factors Fra-1 and JunB are prominent; and 3) Fra-1 expression in breast

tumor cells determines their susceptibility towards doxorubicin. Collectively, these data provide a model whereby FAK-dependent signaling supports the expression of the AP-1 transcription factor Fra-1 and provides survival advantages that protect against therapeutic relevant doxorubicin concentrations, thus appearing a drug-resistant phenotype. These data support the notion that pharmacological intervention of FAK function will provide opportunities for combined therapy in cancer types with increased levels of FAK and chemo-resistance.

Both the orthotopic breast tumor model and the experimental metastasis model indicate that HA-FRNK expression ameliorates *in vivo* resistance against doxorubicin. To our knowledge, this is the first time to show that tumor cell-specific inhibition of FAK enhances drug sensitivity. Although our orthotopic tumor model could not substantiate whether the combined treatment resulted in a complete eradication of subsets of tumor cells or solely a delay in tumor cell proliferation, our experimental lung metastasis model supports that the combined treatment kills off small micro-metastases completely. Thus, the total number of lung metastases decreased, and this was not associated with a clear decrease in the size of the remaining metastases. Importantly, these remaining metastases were in most cases still partly positive for HA-FRNK, indicating that there was not a selection of outgrowth of HA-FRNK negative cells. By microscopic analysis of HA-FRNK stained paraffin sections, no remaining micro-metastases were observed. This suggested that FRNK expression rather supported killing of metastatic cells by doxorubicin, than induced a dormant phenotype of metastasis that formed up till day 12 after injection. Moreover, our *in vitro* data suggest that conditional HA-FRNK expression facilitates the killing of MTLn3 cells by doxorubicin-induced apoptosis and prevents long term colony formation in soft agar assay (data not shown).

We identified Fra-1 as an important regulator downstream of FAK-mediated survival signaling. Thus, both HA-FRNK expression and FAK knock down suppressed *fra-1* mRNA expression as well as protein levels. On its turn Fra-1 knock down sensitized MTLn3 cells towards doxorubicin but did not affect FAK expression. Moreover, Fra-1 overexpression inhibited the onset of apoptosis when FAK was knocked down, but no protection was observed when FAK was expressed. *Fra-1* encodes a leucine zipper protein that can dimerize with proteins of the Jun family and form the transcription factor complex AP-1. Interestingly, JunB was also downregulated after FRNK expression (Fig. 3 and Supplemental Table 2), although this was not significant by qRT-PCR. Based on our microarray data, other Fos and Jun family members have relatively low mRNA expression levels in MTLn3 cells. Therefore, we hypothesize that Fra-1/JunB dimer is an important AP-1 transcription factor complex in these cells. Our previous collaborative work indicates that Fra-1 is the most significant differentially expressed gene among a large panel of human breast tumor cells with either a epithelial or a mesenchymal phenotype, and has an over 600-fold higher average expression level in these mesenchymal-like breast tumor cells (20). MTLn3 cells also have mesenchymal characteristics. Given the fact that mesenchymal-like breast tumor cells typically have a

higher metastatic potential (30), we anticipate that such a metastatic and often drug-resistant phenotype may have a FAK-Fra-1 signaling axis background.

AP-1 is thought to play an important role in the balance between cell proliferation and apoptosis, the response to genotoxic stress and cell transformation. In colon cancer cells, the classical mitogen activated protein kinase pathway through MEK and ERK is essential in the regulation of Fra-1 levels (31). Also stress responses such as DNA-damage by UV and cisplatin, induce an ERK-mediated Fra-1 phosphorylation and stabilization by preventing proteasomal degradation (23, 31). Possibly, the FAK-mediated regulation of Fra-1 in MTLn3 cells is dependent on ERK signaling, although we have not been able to identify a differential activity of ERK upon HA-FRNK expression (data not shown). Fra-1 expression seems crucial in tumor cell invasion and motility. This is related to the inactivation of beta1- integrin in an unknown way (21). The integrin inactivation suppresses the activation of RhoA/ROCK pathway (21). ROCK-mediated contractility is also enhanced upon Fra-1 knock down in MTLn3 cells, without affecting the expression of FAK itself. This is associated with larger and less dynamic focal adhesions. Interestingly, doxorubicin itself increases the contractility of MTLn3 cells in a ROCK dependent manner; however ROCK inhibition does not affect the doxorubicin-induced apoptosis (data not shown). Since Fra-1 knock down decreases the dynamics of focal adhesion and most likely also focal adhesion derived (survival) signaling, we anticipate that focal adhesion stabilization may be the driven force for sensitization towards doxorubicin. Indeed, FAK-mediated signaling via the PI-3K/AKT pathway seems important to control doxorubicin-induced apoptosis in MTLn3 cells (7). However, we can not exclude other possible mechanisms by which Fra-1 in concert with focal adhesion signaling converges to modulate drug-resistant phenotype. Further research will be essential to unravel the exact mechanism of Fra-1-mediated cell survival in breast cancer cells.

Although we could functionally link modulation of Fra-1 by FAK to drug sensitivity, there are other alternative pathways by which FAK signaling modulates drug resistance and tumor development. Indeed, recently, Halder et al. showed that the strategy with FAK siRNA and docetaxel reduces tumor growth (13). This effect attributed to decreased vascularization with low VEGF levels. In accordance with this, Mitra et al. showed that inhibition of FAK resulted in reduced VEGF expression and smaller tumors in mice (32). However, our microarray analysis did not reveal any HA-FRNK-induced downregulation of VEGF/VEGFR pathway components. Additionally, no difference in the vascularization of primary tumors upon expression of HA-FRNK was observed in the absence and presence of doxorubicin treatment (unpublished results). Also the small size of the micro-metastases at the time point of doxorubicin exposure in our experimental metastasis model in combination with the extensive vascularization of lung tissue makes a relationship between VEGF, angiogenesis and drug sensitization unlikely. Our GO-miner-based gene ontology analysis revealed a clear identification of biological programs that are significantly affected by HA-FRNK expression including *regulation of mitosis*,

mitotic regulation of transcription, regulation of cell growth as well as *cell motility* (Table 1). Defects in such programs are likely to sensitize cells to doxorubicin. It is still questionable whether the gene expression changes are directly affected by HA-FRNK expression or rather indirect consequences of the altered transcription activity of transcription factors such as Fra-1. In addition, some of the most significantly altered genes after HA-FRNK expression (supplemental Table 2) are not included in the gene ontologies. In this respect, it is worthwhile to mention that AKT1 was significantly downregulated by HA-FRNK expression (supplemental Table 2). FAK is involved in the generation of second messengers phosphatidylinositol phosphates (PIP3/PIP2), via recruitment of PI-3 kinase to its phosphorylated tyrosine residue (Y397). This eventually results in the activation of AKT (8, 9). Interestingly, we have previously shown that doxorubicin-induced phosphorylation of AKT1 (PKB) is inhibited by expression of FRNK *in vitro* (7). Therefore, HA-FRNK expression, by disturbing the PI3-kinase pathway, could interfere in an alternative mechanism with tumor cell resistance towards doxorubicin. Further systematic research should be carried out to test all individually downstream candidate effectors of FAK signaling that we have identified.

In conclusion, our data indicate that modulation of FAK *in vivo* can sensitize breast tumor cells towards classical anticancer drugs. Therefore, targeted pharmacological intervention with FAK inhibitors (33) may become an attractive way to combat resistant metastatic breast tumors.

FOTENOTES

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Supplemental Table 1: Experimental set-up for MTLn3-tetFRNK microarrays.

Annotation	Cell line	doxycycline	# of arrays
Tet-on	MTLn3-tet-on	-	3
Tet-on-doxy	MTLn3-tet-on	+	3
Control	MTLn3-tetFRNK	-	3
FRNK	MTLn3-tetFRNK	+	3

Supplemental Table 2: FRNK-induced alterations in annotated genes in MTLn3-tetFRNK cells.

Probe_ID	Gene_Symbol	P-value ¹	FC	Probe_ID	Gene_Symbol	P-value ¹	FC
21856159	Cd3d	0.0010	0.320	22403212	Slc1a7	0.0003	2.703
21936000	Ehhadh	0.0013	0.205	22324795	Ch25h_predicted	0.0008	3.521
22393188	Maf	0.0020	0.536	20943419	Cck	0.0011	3.003
20780557	Havcr1	0.0030	0.452	21321652	DOXL1	0.0015	1.946
21031326	Slc17a1	0.0032	0.453	22327267	Bcl2l12_predicted	0.0015	2.591
21201447	ErbB3	0.0045	0.490	21726405	Csf3r_predicted	0.0017	2.358
20762938	Egfl9_predicted	0.0052	0.548	21267977	Igsf4b_predicted	0.0023	3.663
21336348	Dhrs9	0.0053	0.493	21927201	Olr1468	0.0027	3.226
21784618	Il16	0.0054	0.377	20763458	Crebl2_predicted	0.0030	2.114
21849152	F2r	0.0059	0.597	20752903	Cd7_predicted	0.0034	2.079
21748787	Tnks_predicted	0.0060	0.450	21979282	Ndr4	0.0034	2.000
21743859	Eif2c2	0.0064	0.605	21770029	Slc34a3	0.0038	2.532
21788095	Prkaca	0.0065	0.564	21476914	Cldn23_predicted	0.0042	1.931
21282318	Olr859_predicted	0.0078	0.342	20879943	Pgam2	0.0047	1.890
20788184	Pigm	0.0079	0.566	22014663	Sirt1_predicted	0.0053	1.761
21940318	Wisp1	0.0094	0.611	20777806	Tcf15_predicted	0.0055	2.155
21231240	Gmps_predicted	0.0094	0.591	21631081	Cyp2a2	0.0064	3.571
21565791	LOC361237	0.0098	0.573	21215224	Pias3	0.0081	2.849
21876989	Vat1_predicted	0.0101	0.601	20930584	Ubt1	0.0084	2.375
22227921	Kptn_predicted	0.0103	0.507	22391474	Selenbp1	0.0086	1.953
21105219	Cdc25b	0.0105	0.500	20991663	Pfas_predicted	0.0092	1.642
21998913	Dapk2_predicted	0.0105	0.586	21276979	Sftpd	0.0138	1.761
20779692	Zfp238	0.0106	0.450	22395853	Sox18_predicted	0.0144	1.859
22381611	Dlc2	0.0116	0.585	21569506	Palmd_predicted	0.0155	1.880
21574843	Slk	0.0127	0.614	21848369	Cacna1a	0.0157	2.381
21944508	Axl_predicted	0.0133	0.569	21507225	Ka40	0.0160	2.020
21503808	Fbxl12_predicted	0.0133	0.614	20990619	Olr567_predicted	0.0161	1.845
21200143	Slc35d1_predicted	0.0141	0.619	21960505	Ghr	0.0161	1.742
20880470	Mapre3	0.0142	0.561	21761328	C1qb	0.0167	1.672
21600070	Mrps10_predicted	0.0146	0.660	20700469	Pnrc1	0.0169	1.721
21270267	Akap1	0.0151	0.649	21136726	Guca2a	0.0172	1.661
20910301	Slc25a14	0.0152	0.597	20846089	Thea_predicted	0.0177	1.639
22262479	Tle4	0.0153	0.556	21997485	Rtn3	0.0180	2.899
21944206	Pik3c2b_predicted	0.0154	0.613	20905752	Cplx1	0.0183	2.183
21075257	Cask	0.0159	0.518	21847074	MGC72957	0.0190	1.953
22120455	AKT1	0.0159	0.472	22126943	Aqp6	0.0209	1.558
21545404	Ppt2	0.0167	0.587	21142883	Dhrs3_predicted	0.0212	1.667
21310129	Ok138	0.0168	0.633	21255269	Pygm	0.0221	2.183
22135944	Gna12	0.0174	0.556	22287230	Lin10	0.0224	2.273
21742615	Rrm1	0.0178	0.154	20997792	Tagln3	0.0231	1.799
21548145	Slc16a2	0.0180	0.447	21902211	Hbp1	0.0237	1.661

22289377	Ptpn12	0.0185	0.555	20794054	Mfap3	0.0254	2.336
21661580	Hk2	0.0188	0.623	21137211	Wfdc1	0.0255	1.558
20828341	Acs11	0.0188	0.621	21325545	Mmp15_predicted	0.0266	1.739
21978152	Cln5_predicted	0.0189	0.652	22267496	RGD1306702_predicted	0.0267	1.773
21833350	LOC286990	0.0190	0.533	20859138	Aldh3a1	0.0276	1.613
21400107	Rab40c	0.0193	0.550	21379704	Kns18_predicted	0.0282	1.603
21851217	Card10_predicted	0.0193	0.608	21003922	Cabc1_predicted	0.0289	1.560
21959286	Taf1a_predicted	0.0208	0.615	22117376	Usp30_predicted	0.0292	1.639
21159331	Oit3	0.0209	0.639	22373953	LOC60665	0.0299	1.792
22407543	Ggcx	0.0213	0.521	21315820	RGD1308955_predicted	0.0304	1.736
21155260	Nucb2	0.0216	0.445	22351471	RGD1309459_predicted	0.0321	1.721
21010472	Usp38_predicted	0.0221	0.657	20743259	Cda08	0.0325	2.083
20821908	Myh9	0.0222	0.637	22120457	RGD1311107_predicted	0.0326	1.912
21372799	Il17f_predicted	0.0225	0.529	22372665	Exo1_predicted	0.0327	1.786
22243043	RGD1310520_predicted	0.0231	0.602	21035433	Ccl20	0.0329	1.795
21281328	Cav2	0.0234	0.648	20904552	Cldn15_predicted	0.0345	2.463
20824132	Ankrd1	0.0241	0.625	20888760	Olr1337_predicted	0.0347	1.718
21603659	Slc17a6	0.0242	0.562	21220396	Ttc16_predicted	0.0347	1.618
21535209	Cyp3a13	0.0243	0.644	21570101	Cbll1_predicted	0.0349	1.592
21622250	Thap6_predicted	0.0244	0.483	20737316	Gpr124_predicted	0.0355	2.268
21876288	Lrp8_predicted	0.0249	0.624	21306709	RGD1306952_predicted	0.0361	1.957
22139518	Etsrp71_predicted	0.0249	0.581	21954151	Ccng2_predicted	0.0374	1.686
22336310	Junb	0.0251	0.639	22097357	MGC94550	0.0375	1.520
22136467	Phf15_predicted	0.0273	0.458	22358977	Hapln4_predicted	0.0410	1.876
21314484	Lgals9	0.0276	0.630	21849031	Cox6c	0.0422	1.730
21160091	Slc4a11_predicted	0.0279	0.631	20876655	Nr1h4	0.0441	1.901
22282806	Lrrc15	0.0282	0.323	21417467	Cacna1b	0.0446	1.538
21305806	Fos11	0.0284	0.564	22175193	Wt1	0.0447	1.901
21219586	Pou2f2	0.0287	0.656	21176640	Tpbg	0.0465	1.536
21897732	Stambp	0.0291	0.658	20854804	Ka17	0.0470	1.499
20876299	Rnf40	0.0293	0.644	20868496	Sod2	0.0475	1.667
21006903	Exoc8	0.0301	0.639	20737825	Notch4	0.0476	1.577
21588690	Nefh	0.0307	0.576	20752295	Tep1	0.0479	1.555
21968384	Fcnb	0.0308	0.540	21584298	Dpp8_predicted	0.0482	1.504
22207768	Mdh1b_predicted	0.0313	0.400	21549591	Siah1a	0.0495	1.580
21110267	Madh3	0.0313	0.456				
21890138	Znf629_predicted	0.0323	0.559				
22288872	Gk-rs1_predicted	0.0324	0.571				
21404310	Hnrpr	0.0332	0.656				
21875776	Nmt2	0.0337	0.665				
21137506	Fkbp14_predicted	0.0341	0.612				
21233599	Tcfe2a	0.0347	0.473				
20875200	Np	0.0353	0.574				
20826517	Meox1_predicted	0.0361	0.353				
20780470	Pim1	0.0367	0.641				
21952268	Klre1	0.0371	0.574				
20989100	Ppargc1a	0.0373	0.590				
21929390	Lgals5	0.0381	0.629				
21113865	Il24	0.0382	0.556				
21985561	Lcn7	0.0385	0.655				
20862355	Rufy1_predicted	0.0388	0.574				
21396873	Ctrl	0.0389	0.650				
21620770	Nppb	0.0389	0.495				
22067183	Epn2	0.0392	0.656				

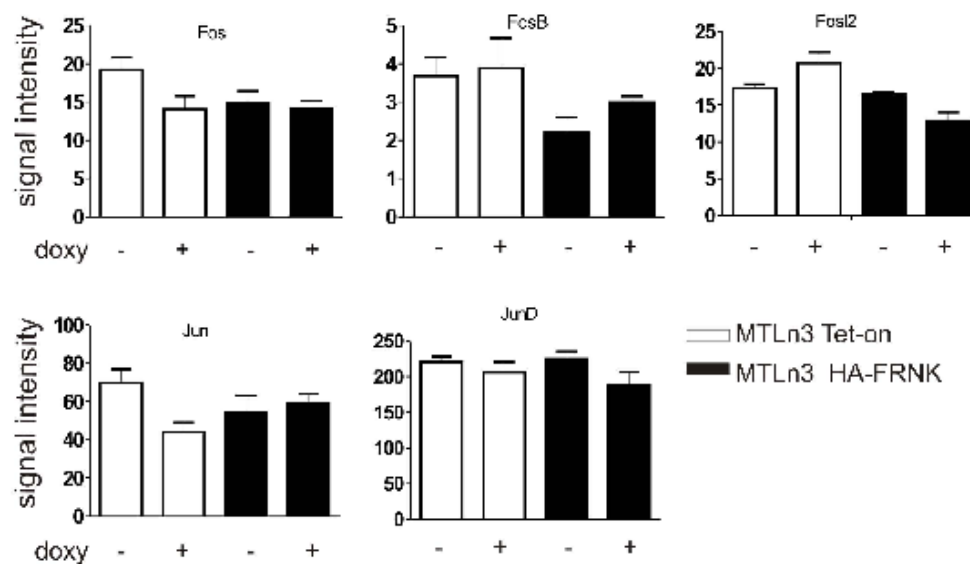
21591414	RGD1305424_predicted	0.0402	0.404
21132402	Phospho1_predicted	0.0405	0.667
21154733	Npl4	0.0406	0.635
22035995	Fgd3_predicted	0.0407	0.642
21481266	Dnaja2	0.0415	0.626
22066307	Actn4	0.0422	0.661
21239072	Fbxl14_predicted	0.0423	0.667
21288312	Upb1	0.0431	0.575
21549672	Hrasls3	0.0431	0.568
21289633	Dbt	0.0432	0.477
21466445	Synj2	0.0434	0.644
21271425	Tufm_predicted	0.0441	0.647
21995038	Phlda1	0.0443	0.662
21497995	Procr_predicted	0.0446	0.666
21388641	Hmmr	0.0453	0.667
20884920	LOC493574	0.0458	0.623
21378546	Tnpo2_predicted	0.0459	0.604
21535218	Car7_predicted	0.0467	0.518
21418355	Mgat5	0.0473	0.611
22413236	Pdrp	0.0478	0.656
21033847	Rap2b	0.0479	0.617
21513568	Loxl2_predicted	0.0480	0.640
21921832	Clc	0.0482	0.648
22104079	Abhd2_predicted	0.0482	0.631
21700036	Tagln	0.0485	0.407
21204716	Slc4a2	0.0491	0.648
21918636	Pdlim7	0.0493	0.510
21667412	Mef2d	0.0493	0.614
21153498	MGC94736	0.0497	0.639

¹Selection of annotated genes is based on the following criteria: $P < 0.05$ and Fold Change (FC) > 1.5 or < 0.67 ; Left column: down-regulated genes; Right column: up-regulated genes. Lists are sorted by P-values.

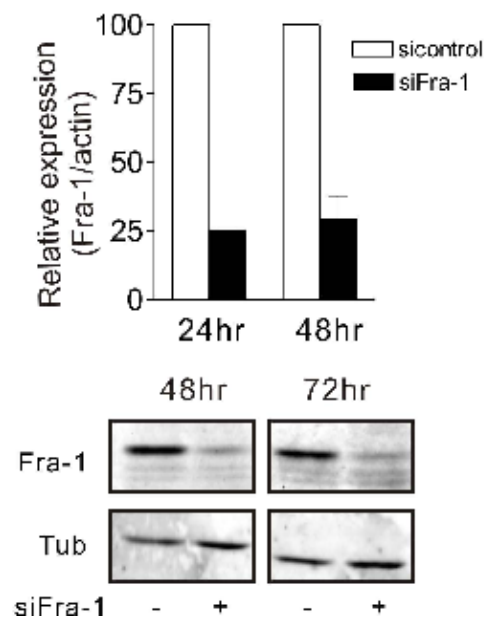
Supplemental Figure 1: HA-FRNK-dependent gene expression of Fos and Jun family members. Shown are the expression values of other Fos and Jun family members in MTLn3 Tet-on and MTLn3-tetFRNK cells treated with or without doxycycline, as determined in the microarray experiments.

Supplemental Figure 2: Validation of siRNA-mediated Fra-1 knock down. MTLn3 cells were transfected with siFra-1 and *fra-1* mRNA was evaluated after 24 and 48 hr by qRT-PCR using actin as an internal control (upper panel). Fra-1 protein expression was estimated with immunoblotting after 48 and 72 hr by western blot (lower panel).

Supplemental figure 1.



Supplemental figure 2.



CHAPTER 4

c-Jun N-terminal Kinase coordinates vincristine-induced Rho-kinase-dependent cell contractility through the focal adhesion-associated scaffold protein paxillin

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Running title: a paxillin-JNK linkage in the vincristine stress response

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ABSTRACT

Microtubule disrupting agents cause cell cycle arrest at G2/M transition as well as actin cytoskeleton reorganization, focal adhesion stabilization, cellular contraction and cell rounding prior to apoptosis. Here we used MTLn3 mammary adenocarcinoma cells to study the role of the c-jun N-terminal kinase (JNK) signaling pathway and its substrate paxillin on vincristine-induced actin network and focal adhesion reorganization. Vincristine caused G2/M arrest in association with actin stress fiber formation and focal adhesion stabilization later followed by apoptosis. This was accompanied with an early activation of JNK at focal adhesions. Inhibition of JNK with SP600125 inhibited actin stress fiber, focal adhesion formation and cell rounding but not cell cycle arrest. Vincristine caused JNK dependent phosphorylation of paxillin on serine residue 178 as well as another posttranslational modification of paxillin, which was independent on serine178 phosphorylation. While the inhibition of Rho-kinase prevented vincristine-induced myosin light chain phosphorylation and actin stress fiber formation, it did not affect JNK activation, paxillin modification or cell cycle arrest. Paxillin knock down inhibited vincristine induced c-Jun activation, myosin light chain phosphorylation, actin stress fiber formation and focal adhesion stabilization but not JNK activation. Altogether our data indicate an important role of the JNK-paxillin axis in vincristine-induced actin stress fiber formation and cell contractility, independent from cell cycle arrest.

INTRODUCTION

Cell adhesion is required for diverse cellular processes in both physiological and pathological conditions such as tissue regeneration, tumor cell invasion and metastasis formation as well as cellular responses to cell injury directly linked to the control of apoptosis. The dynamics of cell adhesions involve a continuous remodeling of F-actin cytoskeletal network, which must be coordinated both spatially and temporally to generate the correct biological outcome. Diverse cellular stress conditions, such as ATP depletion (1, 2), oxidative stress (3), mechanical stress and exposure to different chemicals and anticancer drugs, cause the disorganization of actin cytoskeleton, which is often associated with cell death (4). The exact molecular mechanisms of reorganization of F-actin network and its relationship to apoptosis upon cellular stress are largely unknown.

Microtubule disrupting agents (MDAs) including vinca-alkaloids are an important class of anticancer drugs in the treatment of a variety of cancers (5-7). Vinca-alkaloids, such as vincristine and vinblastine, cause complete microtubule depolymerization. Besides the induction of cell cycle arrest and apoptosis in a variety of cell types (8-10), microtubule destabilization has the unique feature to induce an increased formation of F-actin cytoskeleton network and focal adhesions, which is in accordance with increased cellular

contraction, a phenomenon observed during apoptosis due to caspase-dependent activation of Rho kinases (11, 12). So far the relationship between actin organization, cell cycle arrest and/or apoptosis caused by microtubule disruption has not been investigated. Moreover, it remains largely unclear what signaling events initiate cellular contractility upon microtubule depletion.

Focal adhesions are dynamic multiprotein complexes that form the closest contacts between cells and extracellular matrix. They consist of a variety of signaling, adaptor and cytoskeletal proteins that mediate downstream signaling for cell survival, proliferation and migration. Apoptosis induced by a range of agents is associated with the dephosphorylation, phosphorylation and/or degradation/cleavage, of various focal adhesion-associated proteins, such as focal adhesion kinase (FAK) and the cytoskeletal scaffold protein paxillin. This is often preceded by the formation of stress fibers and focal adhesions (11, 13, 14). Also, vinblastine causes increased F-actin stress fiber formation in endothelial cells, which is associated with ROCK dependent myosin light chain (MLC) phosphorylation (15). Activation of the RhoA/ROCK pathway causes increased actin/myosin driven bundling of F-actin filaments and focal adhesion formation (16).

Diverse MDAs, including vincristine and taxol, cause the drastic activation of c-Jun N terminal kinase (JNK) through ASK1- and Ras mediated signaling (17), or other MAPK family members like ERK and p38 (18). Activation of JNK is involved in the early apoptosis caused by microtubule disrupters (18, 19) and seems independent on microtubule disruption-related phosphorylation of Bcl-2 and Bcl-XL (20, 21). Recent data indicate that active phosphorylated form of JNK accumulates at focal adhesions in several cell types, especially under cellular stress conditions (22, 23). This may be via the binding of JNK adaptor protein JSAP1 to FAK at focal adhesions (24-26), or an upstream kinase of JNK, MEKK1 downstream of FAK (27). Besides, JNK can phosphorylate the focal adhesion adaptor protein paxillin at serine residue 178 after epidermal growth factor signaling (28). These combined observations suggest a potential relationship between microtubule disruption-induced JNK activation, modification of focal adhesion-associated proteins and cell contraction possibly in direct relation to the onset of cell cycle arrest and/or apoptosis.

In this paper, we have used mammary adenocarcinoma cell line MTLn3 to determine the relationship between F-actin reorganization, JNK activation and actin/myosin-based cell contraction caused by the anticancer vinca alkaloid drug vincristine. We show that vincristine causes early cell cycle arrest in association with a rapid appearance of actin/myosin-based cell contraction and focal adhesion formation, which is followed by cell rounding. The actin stress fiber formation is accompanied by two modifications of paxillin which are dependent on JNK activation: serine178 phosphorylation and a yet undefined modification that causes an electric mobility shift. SiRNA mediated knock down of paxillin inhibits vincristine-induced JNK pathway, stress fiber formation and cell contractility. Moreover, the inhibition of JNK retards cell contraction, and focal adhesion

formation. These events are dissociated from vincristine-induced cell cycle arrest. Our data support a model whereby the JNK-paxillin axis plays a crucial role in microtubule disruption-induced stress response related to cellular contraction but not cell cycle arrest.

EXPERIMENTAL PROCEDURES

Materials - Alpha-modified minimal essential medium with ribonucleosides and deoxyribonucleosides (α -MEM) and fetal bovine serum (FBS) were from Invitrogen. Bovine serum albumine (BSA), p38 inhibitor SB203580, phosphoinositide-3 kinase inhibitor wortmannin, bisindolylmaleimide I, propidium iodide (PI), protein G-Sepharose, vincristine, 7-amino-4-methylcoumarin (AMC) and RNase A were from Sigma. SP600125 was from Biomol International. Y27632 was from Tocris Bioscience. Rat tail collagen type I was from Upstate. MEK inhibitor U0126 was from Promega and H89 was from Calbiochem. All other chemicals were of analytical grade.

Cell culture and stable cell lines - MTLn3 rat mammary carcinoma cells were cultured as described (13). For experiments, cells were plated on dishes or collagen-coated (20 μ g/ml) coverslips and grew in complete medium for three days. Cells were exposed to vincristine in α -MEM supplemented with 2.5% FBS for the indicated periods; in some experiments cells were pretreated with pharmacological inhibitors for 30 min.

To generate stable cell lines, MTLn3 cells were transfected with GFP-paxillin or GFP-paxillinS178A in vector (0.72 μ g) along with empty vector pcDNA3 (0.08 μ g) using LipofectAMINE Plus reagent according to manufacturer's procedures. Stable transfectants were selected using geneticin (G418, 600 μ g/ml; Invitrogen). Individual clones were picked and maintained in complete medium containing 100 μ g/ml G418. Clones were analyzed for the expression of the GFP constructs by flow cytometry analysis, western blotting and immunofluorescence.

Transient transfection - GST-wt-SEK1 and GST-DN-SEK1 (kindly provided by John Kyriakis) were transiently cotransfected together with GFP-paxillin with LipofectAMINE Plus (Invitrogen) for 48 hr. For knock down experiments, cells were transfected with SmartpoolTM siRNA against rat paxillin (5-50 nM) using Dharmacon reagent 2. siRNA against GFP was used as a control. All experiments after siRNA transfection were performed between 48-72 hr after transfection.

Cell cycle and apoptosis analysis - Cell cycle distribution and apoptosis were determined with cell cycle analysis as described before (29) using flow cytometry (FACS-Calibur, Becton Dickinson). The percentages of cells in sub-G₀/G₁, G-, S- and M-phase were determined with Cellquest software (Becton Dickinson). SubG₀/G₁ represents the apoptotic fraction (30). Caspase activity assay was performed with Ac-DEVD-AMC as described before (29, 30), the release of AMC was measured on a fluorescence plate

reader (HTS 7000 Bio assay reader, Perkin Elmer Life Sciences). Caspase activity was calculated as pmol AMC/ (min*mg protein) using free AMC as a standard.

Gel electrophoresis and immunoblotting - Western blot analyses were done as before (29). Briefly, equal amounts of total cellular protein were separated on a 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 hr at room temperature using I-Block (0.2% casein in Tris-buffered saline with 0.05 % Tween 20 (TBS-T)) for phospho-state specific antibodies or 5% BSA in TBS-T for other primary antibodies. The following primary antibodies were used: anti-tubulin (Sigma), anti-pT180/pY182-p38, anti-pT202/pY204-p44/42 ERK (New England Biolabs), anti-FAK, anti-pY118-paxillin, anti-pY397-FAK, anti-paxillin (Transduction Laboratories), anti-pS178-paxillin (Abcam), anti-pT183/pY185-JNK (Promega), anti-pS63-cjun and anti-pS19-MLC (Cell Signaling Technologies). Incubation with primary antibodies diluted in I-Block or 1% BSA in TBS-T were carried out overnight at 4 °C. Following washing steps, secondary antibodies diluted in either I-Block (GαRb-AP, 1:2500) or TBS-T (GαM-HRP, 1:2000, GαRb-HRP, 1:2000, or GαM-CY5, 1:2500; all antibodies from Jackson) were added for 1 hr at room temperature. After sufficient washes, membranes blocked in I-Block were processed according to the Tropix kit protocol (Applied Biosystems). Membranes blocked in BSA were either developed with ECLplus reagent (Amersham Biosciences) before detection or directly imaged (CY5 staining) on the Typhoon Imager 9400 (Amersham Biosciences).

Immunofluorescence - Immunofluorescence studies were performed as before (31). The following primary antibodies were used: anti-vinculin (Sigma) and anti-tubulin, anti-pY118- paxillin and anti-pY397-FAK, anti-paxillin and anti-pT183/pY185-JNK and anti-pSer19-MLC (Cell Signaling). After three washes in 0.05% Triton/0.5% BSA in PBS (TBP), coverslips were incubated with fluorescent labeled secondary antibodies (Molecular Probes) diluted in TBP for 1 hr at room temperature or overnight at 4°C, followed by two washes in TBP, and one in PBS. Post-fixation was then carried out using 3.7% formaldehyde for 5 min at room temperature. After a final washing step with PBS, coverslips were mounted on glass slides with Aqua Poly/Mount (Polysciences). Cells were visualized on a Nikon E600 fluorescence microscope and a BioRad Radiance 2100 confocal laser scanning system.

TIRF microscopy - Total internal reflection fluorescence (TIRF) microscopy was performed on non-treated or treated GFP-paxillin MTLn3 cells (50 nM vincristine, 8hr) in a climate control chamber. TIRF movies were captured on a Nikon TIRF microscope system (Eclipse TE2000-E, Nikon with automated stage) with framing every 5 minutes for 4 hours using NIS-elements AR software (Nikon).

Statistical analysis - Student's T test was used to determine significant differences between two means ($p < 0.05$).

RESULTS

Vincristine-induced apoptosis of MTLn3 cells is preceded by focal adhesion formation and cell contractility - To investigate the molecular mechanism of microtubule disruption-induced cytoskeleton reorganization and cell contractility, we used the anticancer drug vincristine. Since MDAs also typically induce cell cycle arrest and apoptosis in tumor cells, we first examined the dose-dependent effects of vincristine on cell cycle progression and apoptosis onset. Cells were exposed to increasing concentrations (0-100 nM) of vincristine for 24 hr. The amount of apoptotic cells increased in a dose-dependent manner, and reached a plateau at 50 nM (Fig. 1A, left panel). Vincristine-induced apoptosis (50 nM) was already observed after 8 hr, which further reached a level of 56 % after 16 and 24 hr (Fig. 1A, right panel). The onset of apoptosis was confirmed by a time-dependent (Fig. 1B) and concentration-dependent (data not shown) caspase-3 activation as well as the subsequent cleavage of a caspase 3 substrate, polyADP-ribose polymerase (PARP) (data not shown). The onset of apoptosis at 8 hr was preceded by cell cycle arrest in G2/M phase, which was already initiated 4 hr after exposure (Fig. 1C). For further studies MTLn3 cells were exposed to 50 nM vincristine.

Disruption of microtubule network by the MDA nocodazole induces F-actin stress fiber formation and focal adhesion organization in serum-starved Swiss 3T3 cells (32). Therefore, we determined whether microtubule disruption by vincristine caused similar changes in MTLn3 breast tumor cells. Polymerization of microtubules was completely inhibited by 50 nM vincristine and the microtubule network had virtually collapsed after 4 hours; this was associated with an increase of F-actin stress fiber formation (supplemental data Fig. S1). Vincristine-treated MTLn3 cells appeared to have a more contractile phenotype culminating in cell rounding (Fig. 1D). We reasoned that this vincristine-induced contractile phenotype was associated with enhanced focal adhesion formation. We stained cells for tyrosine phosphorylated paxillin (PY118-paxillin), which specifically localizes at focal adhesions. Untreated cells mainly possessed small focal complexes while occasionally cells with more mature focal adhesions were observed. Vincristine induced the formation of clear focal adhesions that colocalized at the edge of stress fibers at cell periphery (Fig. 1E). Similar observations were obtained after immunofluorescent staining for PY397-FAK and vinculin (supplemental data Fig. S2). Next we visualized vincristine-induced focal adhesion formation in GFP-paxillin MTLn3 cells with TIRF microscopy. In untreated cells the relatively small focal adhesions were highly dynamic. However, in vincristine-treated MTLn3 cells, paxillin accumulated at focal adhesion sites and peripheral ruffles, while focal adhesions became larger in size and less dynamic, followed by cell rounding and apoptosis (supplemental data and movie Fig. S3). Thus, vincristine-induced F-actin stress fiber and focal adhesion formation is an early response that occurs well before the onset of apoptosis.

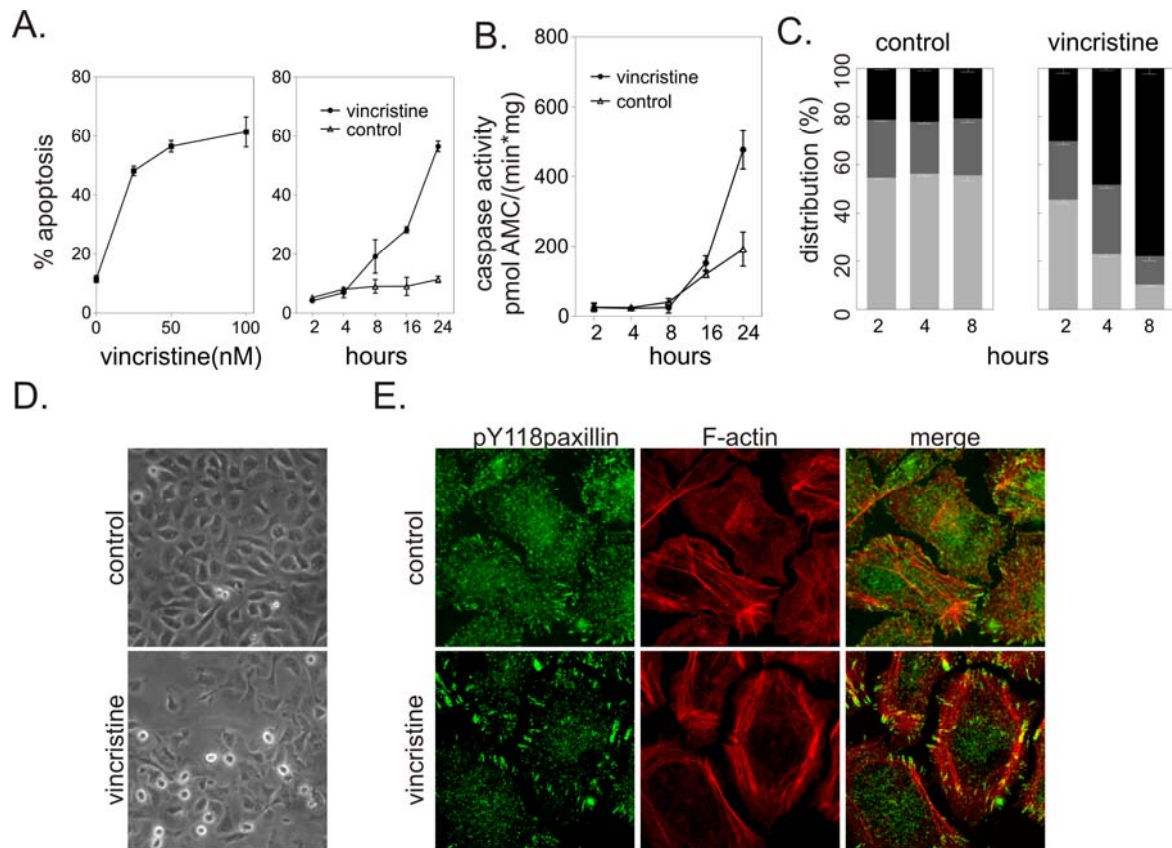


Figure 1: Vincristine causes cell cycle arrest and focal adhesion formation and cell contractility. MTLn3 cells were treated with indicated concentrations of vincristine for 24 hr or exposed to 50 nM vincristine for indicated time periods. Apoptosis was determined by cell cycle analysis and expressed as % subG0/G1 (A). Cells were treated with 50 nM vincristine for indicated time points and caspase activity was measured with Ac-DEVD-AMC. Fluorescent density was visualized on a Fluostar platereader (B). Cell cycle arrest was determined by cell cycle flow cytometric analysis (C). Phase contrast pictures were taken after 8 hr exposure to vincristine (50 nM) (D). Cells were treated with vincristine for 8 hr and fixed and stained with pY118-paxillin and F-actin. The images were acquired on CLSM (E). Data shown are from (A, B and C; mean \pm SEM) or representative for (D and E) three independent experiments.

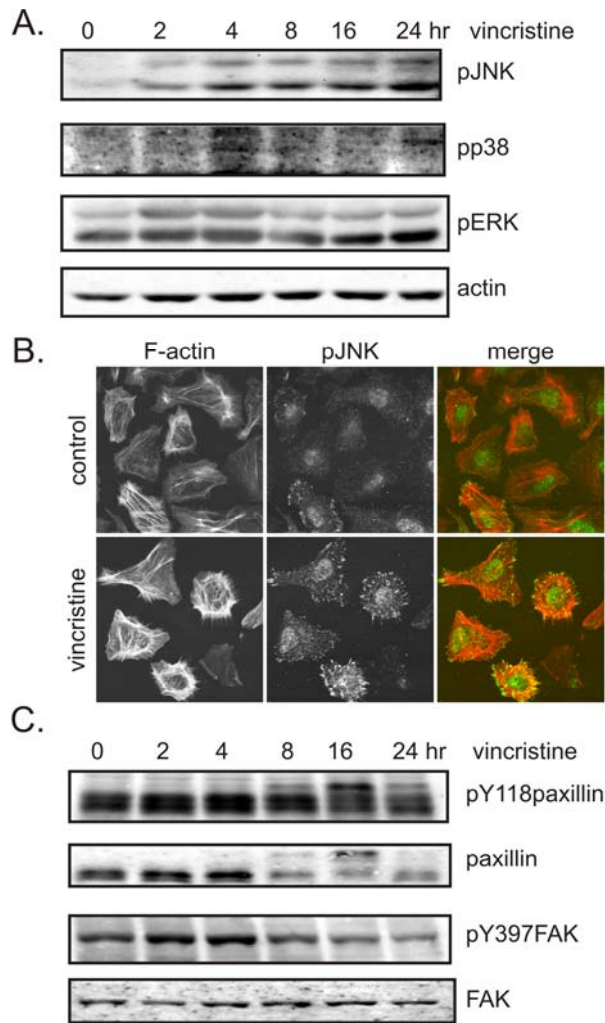


Figure 2: Vincristine induces JNK activation and accumulation at FA sites and reduced mobility shift of the focal adhesion scaffold protein paxillin.

MTLn3 cells were treated with 50 nM vincristine for indicated time points and collected for immunoblotting with antibodies to active forms of MAPKs pERK, pp38, pJNK (A) and FA-associated proteins pY118paxillin, paxillin, pY397FAK, FAK (C). Vincristine-treated cells were fixed and stained for pJNK and F-actin. The images were acquired on CLSM as mentioned in experimental procedures (B). Data shown are representative for three independent experiments (n=3).

Vincristine causes activation of JNK and its localization at focal adhesions in association with a paxillin modification - MDAs cause the activation of stress-activated MAPKs, including JNK and p38, in different cell types (18, 23, 33-36). In order to determine the effect of vincristine on activation of different MAPKs in MTLn3 cells, cells were exposed to vincristine for various time periods followed by western blot analysis of the phosphorylated forms of JNK, p38 and ERK. A clear increase in the phosphorylation of JNK was already evident after 2 hr and reached maximal activation after 8 hr (Fig. 2A). Increased JNK phosphorylation was associated with the phosphorylation of one of its targets, c-Jun (Fig. 3A). No significant increase in ERK phosphorylation occurred after vincristine treatment, while hardly any phosphorylated form of p38 was detectable at any time point (Fig. 2A). In contrast, incubation of MTLn3 cells with hydrogen peroxide did cause a large increase in p38 phosphorylation (data not shown), indicating the functionality of this stress kinase in these cells.

Next, we evaluated the potential relationship between JNK activation and focal adhesion organization in vincristine-induced stress response. The JNK-binding adaptor protein SJAP1 is localized at focal adhesions through an interaction with FAK (25). This results in the localization of the active phosphorylated form of JNK at these sites in different cell types (25, 29). MEKK1, another upstream kinase of JNK, can also bind to FAK (27). Upon vincristine treatment, the active phosphorylated JNK clearly localized at focal

adhesion-like structures at the end of F-actin stress fibers (Fig. 2B). In contrast, in control cells hardly any phosphorylated JNK was associated with focal adhesions, which fits the vincristine-dependent activation of JNK (Fig. 2A). Given the localization of JNK at focal adhesions upon vincristine treatment, we next investigated whether the phosphorylation of two candidate focal adhesion associated proteins, FAK and paxillin, was affected. The expression levels of FAK and paxillin as well as the phosphorylation of FAK at tyrosine residue 397 and paxillin at tyrosine residue 118 were not affected by early phase of vincristine treatment (0-4hr, Fig. 2C). When apoptosis was initiated (see Fig. 1 for comparison), FAK phosphorylation had decreased, which was in agreement with our previous observations in the doxorubicin-induced apoptosis of MTLn3 cells (13). Interestingly, treatment of MTLn3 cells with vincristine caused a clear mobility shift of paxillin already after 4-8 hr (Fig. 2C and see also Fig. 3). Similar observations were obtained at other vincristine concentrations in the range of 10-100 nM. This mobility shift of paxillin suggests a post-translational modification of paxillin in the vincristine-induced stress response.

Vincristine causes JNK-dependent modification of the focal adhesion scaffold protein paxillin - The data described above suggest a possible relationship between paxillin modification and the activation and localization of JNK at focal adhesions. Recent studies indicate that both growth factor and adenoviral E4orf4 protein-induced JNK activation can mediate the phosphorylation of paxillin at serine residue 178 (37). Moreover, the MDA nocodazole causes an increased phosphorylation of paxillin at serine residues in mitotic NIH3T3 cells (38). Therefore, next we evaluated the possible role of JNK in the mobility shift of paxillin by exposing MTLn3 cells to vincristine in combination with a specific inhibitor of JNK, SP600125. Pharmacological inhibition of JNK with SP600125 (0-30 μ M) abolished the mobility shift of paxillin but did not affect the tyrosine phosphorylation of paxillin at tyrosine 118 (Fig. 3A) or FAK at Y397 (data not shown). This effect was also associated with the inhibition of vincristine-induced phosphorylation of the JNK substrate c-Jun. To evaluate the specificity of this effect, we also determined the effect of other protein kinases inhibitors, including U0126 (MEK inhibitor, 10 μ M), SB203580 (p38 inhibitor, 20 μ M), H89 (PKA/ROCK inhibitor, 5 μ M), Y27632 (ROCK inhibitor, 10 μ M), bisindolmaleimide I (PKC inhibitor, 1 μ M) and wortmannin (PI-3 kinase inhibitor, 50 nM). While SP600125 again inhibited paxillin modification, none of the other inhibitors could prevent the reduced mobility of paxillin (Fig. 3B). To further confirm the indispensable role of JNK activation in vincristine-induced modification of paxillin, we transiently co-transfected cells with a dominant negative upstream activator of JNK, DN-SEK1 (GST tagged), together with GFP-paxillin. Vincristine also caused the mobility shift of GFP-paxillin, which was inhibited in cells that co-expressed DN-SEK1 (Fig. 3C). Together, these data indicate that the modification of paxillin is selectively mediated by JNK.

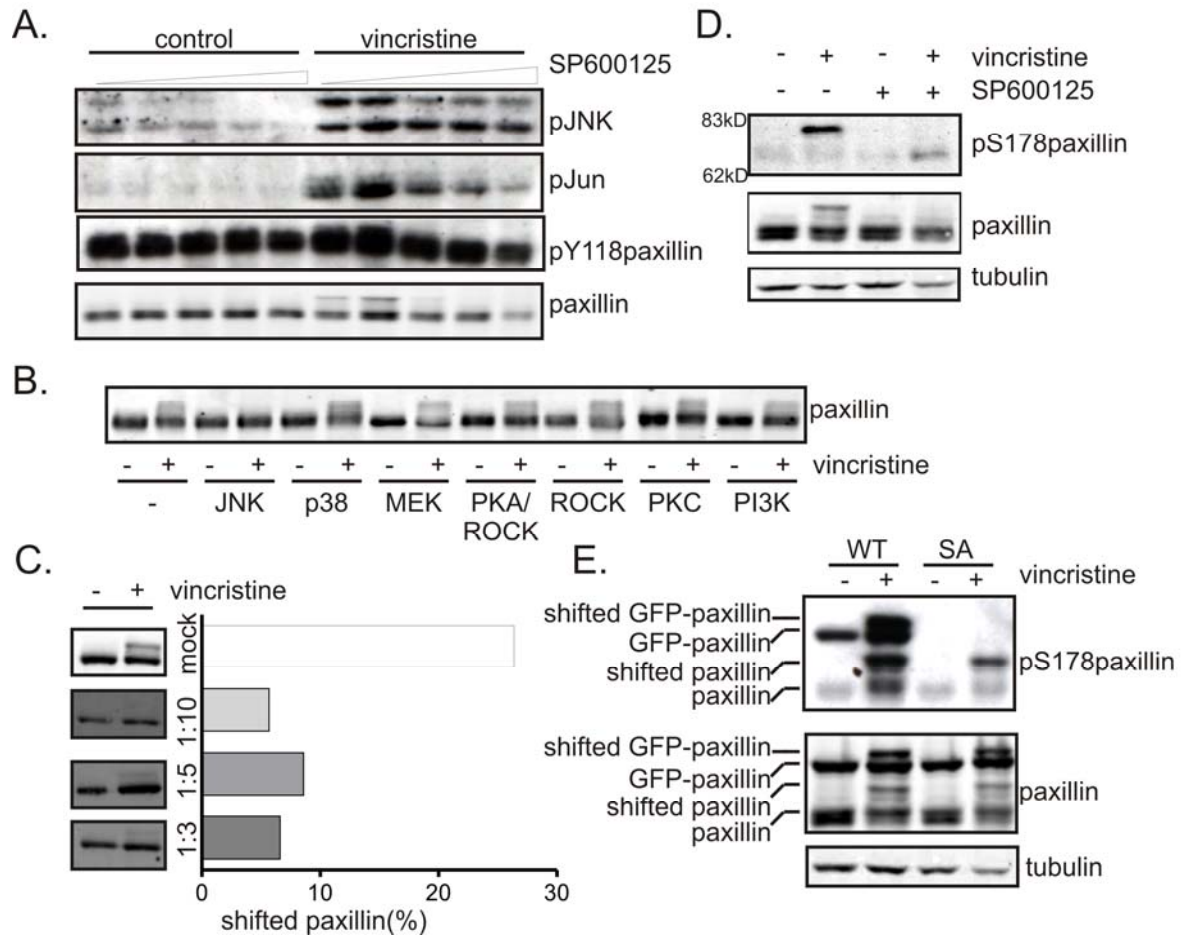


Figure 3: Vincristine causes a selectively JNK-dependent modification of paxillin. MTLn3 cells were exposed to vincristine (50 nM) and JNK inhibitor SP600125 (conc. 0, 5, 10, 20 and 30 μ M) for 8 hr. Cell lysates were collected for immunoblotting with antibodies against pJNK, pc-Jun, pY118paxillin and paxillin (A). Cells were treated with vincristine and various specific inhibitors for individual signal components (JNK, p38, MEK, PKA, ROCK, PKC, and PI3K) and immunoblotted with anti-paxillin (B). Cells were transiently co-transfected with different ratios of GST-tagged DN-SEK1 and GFP-paxillin, followed by immunoblotting for paxillin (left C) and the percentage of shifted paxillin was quantified by densitometry (right C). MTLn3 cells were treated with or without vincristine (50 nM) in the absence or presence of SP600125 (20 μ M) for 8 hr and immunoblotted for pSer178paxillin, paxillin or tubulin (D). GFP-wt-paxillin (WT) and GFP-S178A-paxillin (SA) MTLn3 cells were treated with or without vincristine (50 nM) for 8 hr and immunoblotted for pSer178paxillin, paxillin and tubulin (E). Data shown are representative for three independent experiments (n=3).

Since JNK can phosphorylate paxillin directly at serine residue 178 under certain conditions (39), we next explored the possible modification at this residue after vincristine induced JNK activation. Vincristine caused a drastic phosphorylation of paxillin at Ser178, which was present in the mobility-shifted form of paxillin. Importantly, Ser178 phosphorylation was inhibited by SP600125 (Fig. 3D). Since pSer178-paxillin co-migrated with the mobility-shifted paxillin, we next investigated whether the phosphorylation itself was responsible for the mobility shift. For this purpose we used MTLn3 cell lines stably expressing either GFP-wt-paxillin or GFP-S178A-paxillin mutant. Vincristine caused a mobility shift in both GFP-paxillin and GFP-S178A-paxillin, comparable to endogenous paxillin. However, while phosphorylation of Ser178 was

observed in both GFP-paxillin and endogenous paxillin upon vincristine treatment, no phosphorylation was observed for the GFP-S178A-paxillin mutant (Fig. 3E). This indicates that the JNK-dependent mobility shift of paxillin is not due to phosphorylation at Ser178 site itself, but most likely due to an alternative post-translational modification of paxillin.

Together, these data suggest a model whereby the vincristine-induced activation of JNK and its subsequent localization at focal adhesions mediate the modification of paxillin at phosphorylation site serine 178 as well as another post-translational modification of paxillin which is independent on serine residue 178.

JNK activity is required for vincristine-induced focal adhesion formation and cell rounding in early apoptosis - Given the localization of JNK at focal adhesions and the vincristine-induced modification of paxillin through active JNK, we reasoned that focal adhesion formation, cell contractility and cell rounding were directly related to JNK activity. To evaluate this, MTLn3 cells were treated with vincristine in the absence or presence of SP600125 followed by analysis of focal adhesion and F-actin cytoskeletal organization. SP600125 alone did not alter focal adhesion formation. However, while vincristine caused the presence of bigger focal adhesions at cell periphery, SP600125 inhibited this and still many small focal adhesions were present throughout the cells (Fig. 4A). In addition, overexpression of DN-SEK1, which inhibits JNK activation and paxillin modification (see Fig. 3C), resulted in a decrease in the number of bigger focal adhesions after vincristine treatment (supplemental data Fig. S4). While vincristine-induced stress fiber formation was associated with cell rounding, this was clearly inhibited by SP600125 (Fig. 4C and D). Since focal adhesion formation and stability is essential in cell cycle progression (33, 40), we next determined whether JNK-mediated focal adhesion formation was directly related to vincristine-induced cell cycle arrest. While inhibition of JNK with SP600125 inhibited vincristine-induced cell rounding, it did not affect the onset of vincristine-induced cell cycle arrest at 8 hr prior to apoptosis. Rather, inhibition of JNK with SP600125 itself induced cell cycle arrest in MTLn3 cells (Fig. 4E). Thus, these data indicate that the JNK-mediated paxillin modification, focal adhesion stabilization and cell rounding occur independently from vincristine induced cell cycle arrest. Since SP600125 caused apoptosis of MTLn3 cells at later time points, possibly due to cell cycle arrest, we could not determine the role of JNK in vincristine-induced apoptosis.

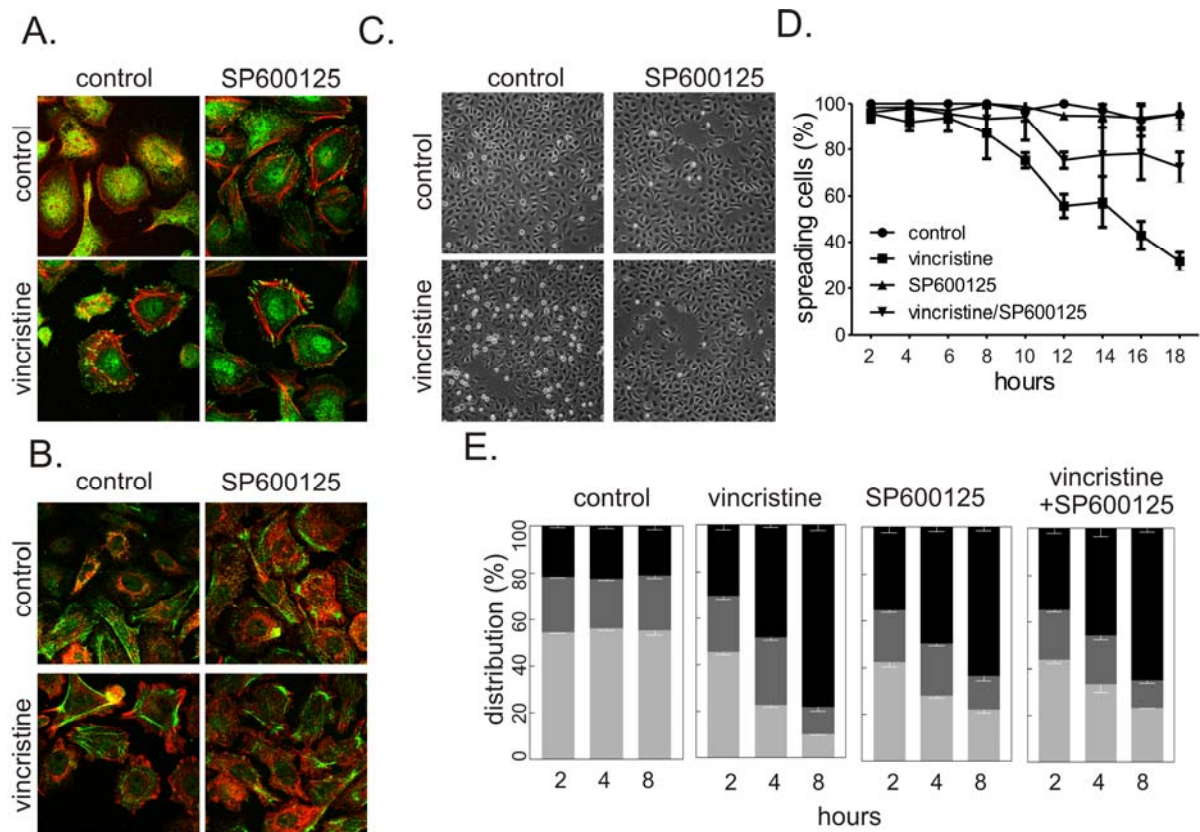


Figure 4: JNK mediates vincristine-induced contractile phenotype but not cell cycle arrest. MTLn3 cells were treated with or without vincristine (50 nM) in the absence or presence of SP600125 (20 μ M) from 2 up till 16 hr as indicated. After 8 hr cells were stained for pY118paxillin (A; green) or pS19-MLC (B; green) together with F-actin (A and B; red). Phase contrast pictures were taken at 8 hr (C). Cells rounding was followed in time and the percentage of cells with rounded morphology was calculated (D; mean \pm SEM; n=3). Cell cycle distribution was determined at the indicated time points by flow cytometry (E; mean \pm SEM; n=3). Images shown are representative for three independent experiments.

Vincristine-induced cell contractility, but not cell cycle arrest and apoptosis, is dependent on ROCK - The data described above suggest a relationship between JNK-mediated stress fiber formation, focal adhesion stability, cellular contractility and vincristine-induced cell rounding. Cell contractility is mediated by the activation of the actin/myosin-based cytoskeletal network through RhoA/ROCK-mediated phosphorylation of the MLC signaling pathway. Therefore we hypothesized that the inhibition of ROCK would prevent vincristine-induced cell contractility. As mentioned above, vincristine caused the association of pS19-MLC with thick F-actin bundles while there was hardly any appearance of p-MLC bundles in non-treated cells. ROCK inhibition with Y27632 prevented the formation of focal adhesions at cell periphery and the formation of the F-actin cytoskeletal network (Fig. 5A). This was associated with the protection against vincristine-induced cell rounding and contractility (Fig. 5B). Next we determined the relationship between JNK activation and ROCK-mediated contractility. Importantly, ROCK inhibition with Y27632 did not affect vincristine-induced activation of JNK and the modification of paxillin (Fig. 5C). Since JNK is involved in F-actin stress fiber formation and focal adhesion stabilization, we hypothesized that JNK activation acts

upstream of ROCK/RhoA pathway. Indeed, the inhibition of JNK with SP600125 inhibited vincristine-induced accumulation of p-MLC at stress fibers (Fig. 4B). Finally, we tested whether ROCK-mediated contractility and cell rounding was not related to vincristine induced cell cycle arrest. Y27632 did not affect cell cycle arrest (Fig. 5E), supporting the dissociation between cell contractility, rounding and cell cycle inhibition. Y27632 did not protect against vincristine-induced apoptosis (data not shown).

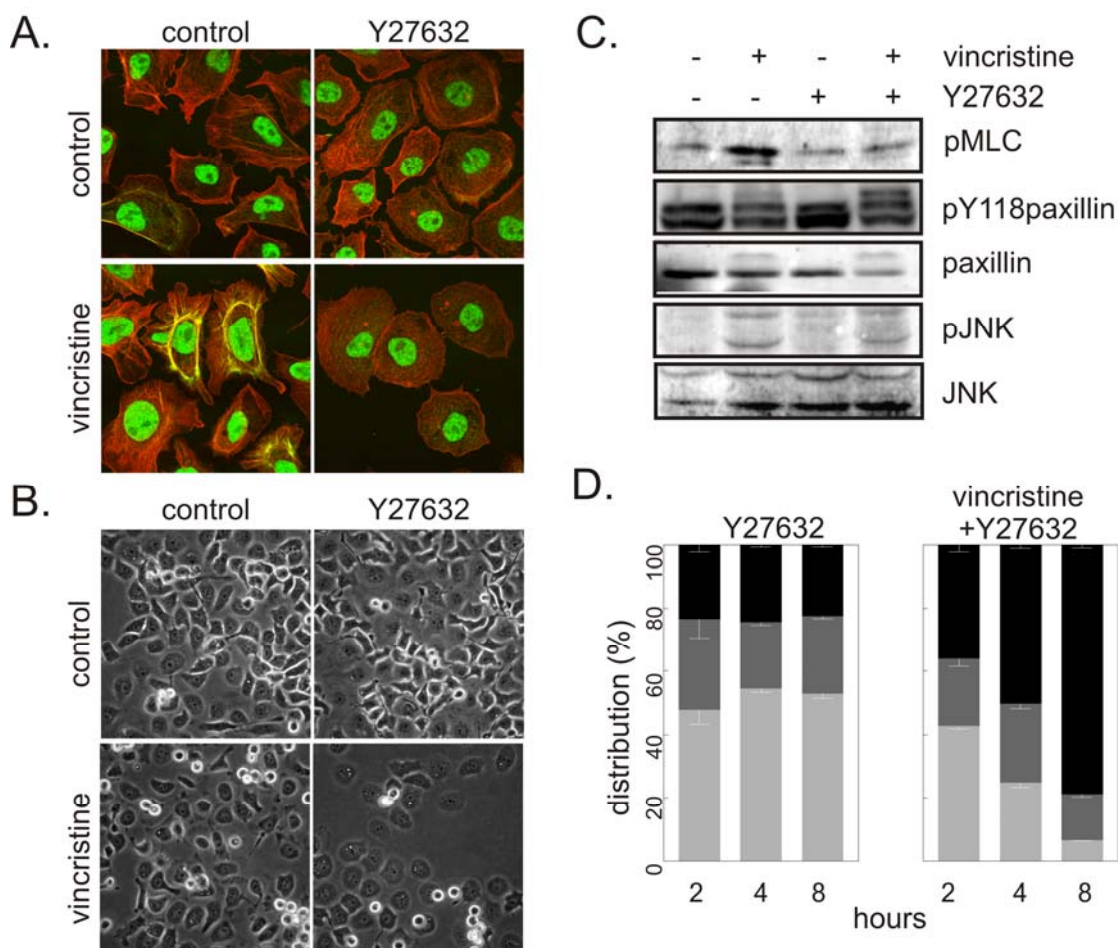


Figure 5: ROCK inhibition prevents vincristine-induced cell rounding and contractility but not cell cycle arrest or JNK activation. MTLn3 cells were treated with or without vincristine (50 nM) in the presence or absence of Y27632 (10 μ M) for 8 hr. pSer19-MLC and F-actin were stained to visualize actin/myosin cytoskeleton (A). Phase contrast pictures were taken for cell morphology (B). 8 hr after exposure, cell lysates were collected and immunoblotted with the antibodies against pY118paxillin, paxillin, pJNK and JNK (C). Cell cycle distribution was determined at indicated time points by flow cytometry (D; mean \pm SEM; n=3; controls were within the same experiments as for SP600125, see figure 4). Images and Western blots shown are representative for three independent experiments.

Paxillin is essential for vincristine-induced focal adhesion formation and cell contractility - The data described above indicate that JNK acts upstream of ROCK to mediate focal adhesion formation and cell contractility after vincristine treatment. Given the fact that paxillin is also modified by JNK in this process, we further investigated the role of paxillin in focal adhesion formation after vincristine treatment. Knock down (KD) of paxillin was achieved by transient transfection with Dharmacon Smartpool siRNA

against rat paxillin (5, 10, 50 nM). Loss of paxillin was already evident at 5 nM siPax; cells with clear paxillin knock down were easily discriminated from non effected cells (Fig. 6A). Maximum KD in ~95 % cells was observed at 50 nM siPax. Paxillin KD did not affect cell survival and cell spreading under normal culturing condition (data not shown). Interestingly, most of the remaining paxillin bound with high affinity at focal adhesion sites (Fig. 6A). Paxillin KD did not affect JNK activation (Fig. 6B). Paxillin KD itself caused some increased staining of pS19-MLC in cells. Nevertheless, while in siGFP-treated cells vincristine clearly increased the percentage of cells with strong pS19-MLC staining, this was not evident in siPax-treated cells (Fig. 6C). Furthermore, to determine whether the paxillin KD inhibited the increased focal adhesion formation by vincristine, we quantified the percentage of cells with enhanced vinculin positive focal adhesions. Indeed, paxillin KD reduced vincristine-induced focal adhesion formation in association with reduced stress fiber formation (Fig. 7). Altogether these data indicate that paxillin is essential for JNK activation after vincristine treatment, whereas JNK mediates downstream phosphorylation and modification of paxillin as well as ROCK-mediated focal adhesion formation and cell contractility.

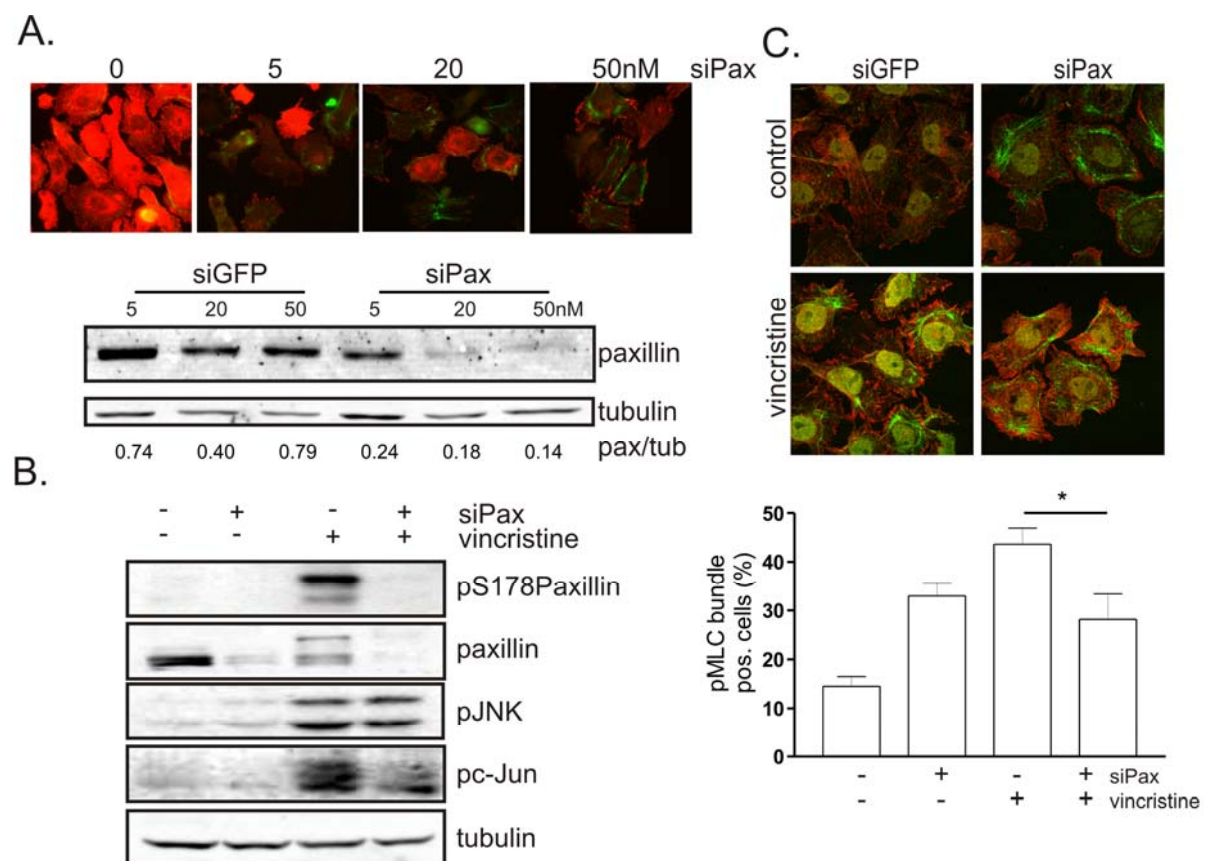
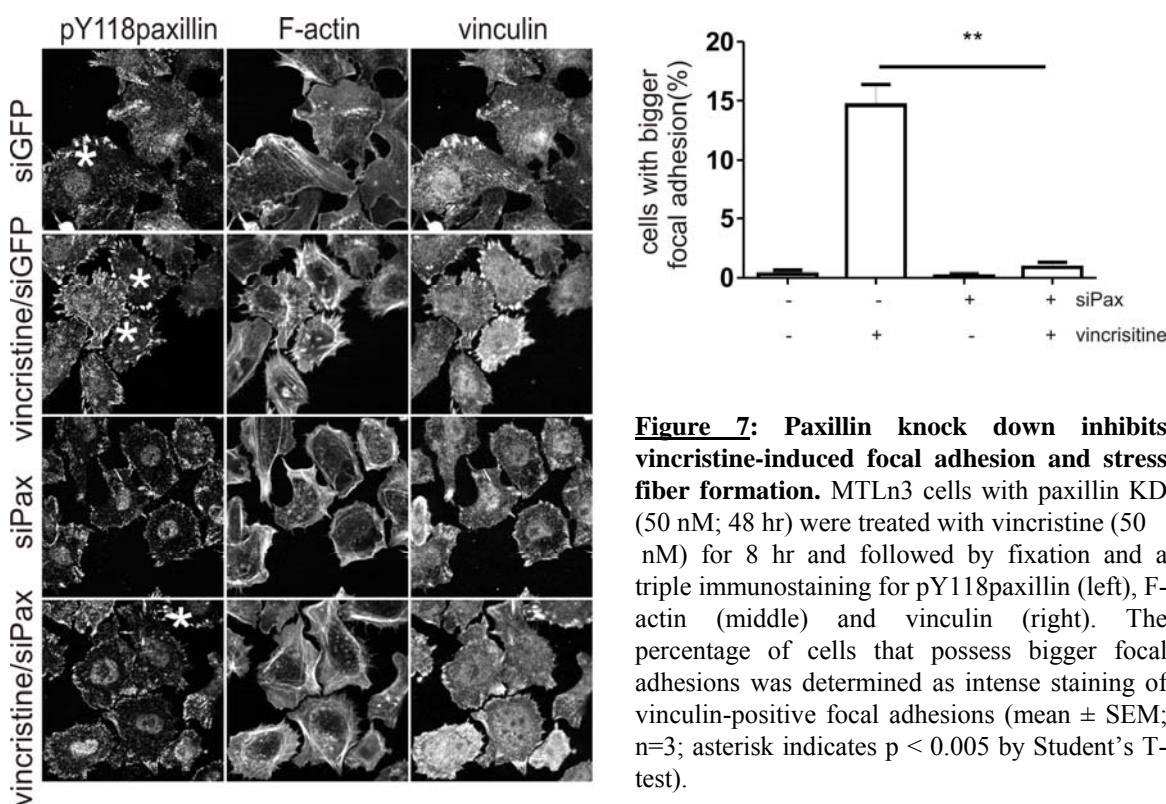


Figure 6: Paxillin knock down inhibits myosin light chain phosphorylation. MTLn3 cells were transfected with 5, 10, 50 nM siRNA smartpool against rat paxillin or GFP (Dharmacon) for 48 hr and followed by immunofluorescent staining against paxillin (top A, red is paxillin; green is pSer19-MLC) and immunoblotting against paxillin (bottom A). MTLn3 cells with paxillin KD (50 nM siRNA) for 48 hr were treated with vincristine (50 nM) for 8 hr and cell lysates were immunoblotted with antibodies against pS178-paxillin, paxillin, pJNK, pc-Jun and tubulin (B), or cells were fixed and stained for pS19-MLC (green) and vinculin (red) (top C). The percentage of cells with strong pS19-MLC along stress fibers were quantified (bottom C; mean \pm SEM; n=3; asterisk indicates $p < 0.05$ by Student's T-test).

DISCUSSION

In the present manuscript we investigated the mechanism by which MDAs affect cell contractility and how this relates to both MDA-induced cell cycle arrest and apoptosis. For this purpose we used mammary adenocarcinoma MTLn3 cell line and the MDA vincristine as a model compound. Our key findings indicate that, firstly, vincristine induced JNK activation is essential in vincristine-induced focal adhesion formation and cell contraction. Secondly, this effect is related to a JNK-dependent modification of the focal adhesion-associated scaffold protein paxillin on two sites, of which one is Ser178 phosphorylation and the other is a Ser178-independent high molecular weight modification. Thirdly, paxillin is not required for vincristine-induced JNK activation but is essential for the enhanced focal adhesion and stress fiber formation in association with the phosphorylation of MLC. Our combined data suggest a model in which vincristine induces JNK activation and its localization at focal adhesions, thereby mediating a post-translational modification of paxillin; the JNK-paxillin activation is essential for the downstream activation of ROCK-dependent MLC which drives the actin stress fiber formation and enhanced focal adhesion formation. This pathway seems independent on vincristine-induced cell cycle arrest and onset of apoptosis.



Our data suggest that vincristine-induced JNK activation directly affects a central focal adhesion-associated adaptor protein paxillin. We observed two different modifications of

paxillin after vincristine treatment: a phosphorylation at Ser178 and a yet unidentified modification. Both modifications were dependent on JNK, since SP600125 inhibited both these modifications. This fits with the co-localization of active JNK and paxillin at focal adhesions after vincristine treatment. Interestingly, Ser178 seems not to be critical for the unidentified modification of paxillin, since GFP-Ser178Ala paxillin could still be modified in a similar JNK-dependent manner. Yet, pSer178-paxillin was primarily present in the shifted form of paxillin, suggesting that Ser178 phosphorylation and the other modification are related. At present we have excluded that this modification is directly due to poly-phosphorylation, ubiquitination or sumoylation (data not shown). Possibly this modification is related to neddylation or glycosylation, which at least is dependent on JNK activity. Importantly, hydrogen peroxide-induced stress response in MTLn3 cells, which induced a drastic but transient JNK activation, was also associated with the same paxillin mobility shift (data not shown), excluding the notion that this would only be observed in the treatment with MDAs. It is noteworthy that another lower migration paxillin family member, possibly paxillin-delta or leupaxin (Mw is ~40kDa), which is recognized by paxillin and pS178paxillin antibodies and also targeted by paxillin siRNAs, responds to vincristine exposure in an exactly similar JNK-dependent pattern in MTLn3 cells (data not shown). Leupaxin shares a large homology with paxillin and forms a complex with the FAK family PYK2, c-Src, PTP-PEST, thereby regulating cell migration, adhesion and invasion (41, 42). It is reasonable to deduce that this paxillin-like protein has a similar role in cytoskeleton reorganization and focal adhesion assembly induced by vincristine. Our data on JNK-mediated phosphorylation of paxillin at Ser178 adds to the lists of both growth factor and xenobiotic-induced JNK-dependent phosphorylation of paxillin (21, 28, 36, 43). Regardless of the actual type of post translational modification of paxillin in our model, the data support the general perception that JNK activation has many different downstream effectors which may be involved in specific biological responses related to either cell stress or other physiological conditions. In this respect we also observed that EGF and HGF induce a JNK-dependent phosphorylation of paxillin, although this is not the same mobility shift of paxillin by vincristine (Le Devedec et al, manuscript in preparation).

The modulation of paxillin in focal adhesion turnover is not only due to phosphorylation at Ser178, but also other sites, such as Ser273 via p21-activated kinase activity. Ser273 is important to localize paxillin in a GIT1-PIX-PAK complex and regulate adhesion and protrusion dynamics (44, 45). Other serine residues like Ser126 and 130 of paxillin can be phosphorylated in an ERK-dependent manner and this is also involved in cytoskeleton reorganization (46). In our hands ERK was not activated by vincristine, and U0126 did not affect the mobility shift of paxillin, excluding the likelihood of enhanced phosphorylation of these sites after vincristine treatment.

Our data provide a link between JNK-mediated paxillin modification and increased focal adhesion formation in association with activation of actin/myosin-based cell contractility. All these data indicate that the inhibition of JNK with SP600125 inhibits focal adhesion

formation, pSer19-MLC positive stress fibers and cell rounding. Similarly, knock down of paxillin inhibits these vincristine-induced events. Since paxillin does not affect vincristine-induced JNK activation, we anticipate that JNK acts upstream of paxillin. At this moment we do not know whether paxillin Ser178 phosphorylation and/or the alternative modification of paxillin, both of which are mediated by JNK activation, are central in vincristine-induced focal adhesion formation and cell contractility. We expressed Ser178Ala-paxillin mutant in MTLn3 cells; these cells show decreased focal adhesion dynamics, are rounded up and have difficulties to fully spread upon stimulation with EGF (Le Devedec et al. in preparation). These results would argue that Ser178 phosphorylation is involved in cell spreading process rather than promoting cell contractility process, and would leave the other modification of paxillin as the major mediator of cell contractility. Identification of this modification will be an important step to further understand the role of paxillin in cellular stress response.

Our data suggest that JNK and paxillin act upstream of ROCK-dependent actin/myosin-mediated cell contractility. As indicated above, irrespective of the actual type of paxillin modification, paxillin KD resulted in a similar inhibition of vincristine-induced phenotype as did SP600125. Both JNK inhibition and paxillin knock down prevented vincristine-induced MLC phosphorylation. This was also inhibited by an inhibitor of ROCK, Y27632, but ROCK inhibition did not affect JNK activation and paxillin modification. A JNK/paxillin/ROCK relationship has also been indirectly shown in another recent manuscript, indicating that JNK and pSer178-paxillin are essential in the recruitment of paxillin to FAs from an internal pool in a ROCK dependent manner (37). How could the JNK-paxillin linkage lead to enhanced ROCK-mediated pSer19-MLC and cell contraction? It is known that other MDAs lead to the activation of RhoA or a shift in the balance between pro-motile Rac activity and pro-contractile RhoA activity (47). Possibly this is related to the disrupted targeting of active Rac1 molecules to focal adhesions through the microtubular tips, since vincristine disrupts this process. In our hands the expression of active Rac mutants prevented vincristine induced contractility (data not shown). The association of paxillin with the GIT1-PIX-PAK complex is important for the regulation of cell adhesion and protrusion dynamics through Rac activation (44). Possibly, the JNK-dependent paxillin modifications affect the formation of this complex and the localized Rac activation that would otherwise promote lamellipodia formation and antagonize cell contractility.

The JNK-mediated contractility seems not to affect vincristine-induced cell cycle arrest, as neither SP600125 nor Y27632 inhibited cell cycle arrest. Cell cycle arrest is most likely due to disrupted microtubule and spindle formation. Also the onset of apoptosis was not affected by preventing cell contractility (data not shown). Therefore, we propose that, at least in our *in vitro* model, vincristine-induced cell cycle arrest and apoptosis are distinct from cytoskeletal reorganization. Since the cytoskeletal events occur at equimolar concentration as the microtubule-disrupting activity of vincristine (47), one could anticipate that under conditions of *in vivo* vincristine treatment regimens, vincristine may

not only prevent cell cycle progression, but also limit the efficiency of tumor cells to migrate and intravasate due to decreased focal adhesion dynamics. This would prevent the dissemination of metastatic tumor cells, and would be an additional advantage of MDA therapy.

In conclusion, we demonstrate the role of JNK-paxillin axis in the regulation of ROCK-dependent actin/myosin-related tumor cell contractility after vincristine treatment. Although the modulation of JNK-paxillin axis in tumor cells might be relevant to target cancer progression, further research is required to identify the exact paxillin modifications and the downstream effectors of the JNK-paxillin signaling hub.

FOOTNOTES

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Fig.S1

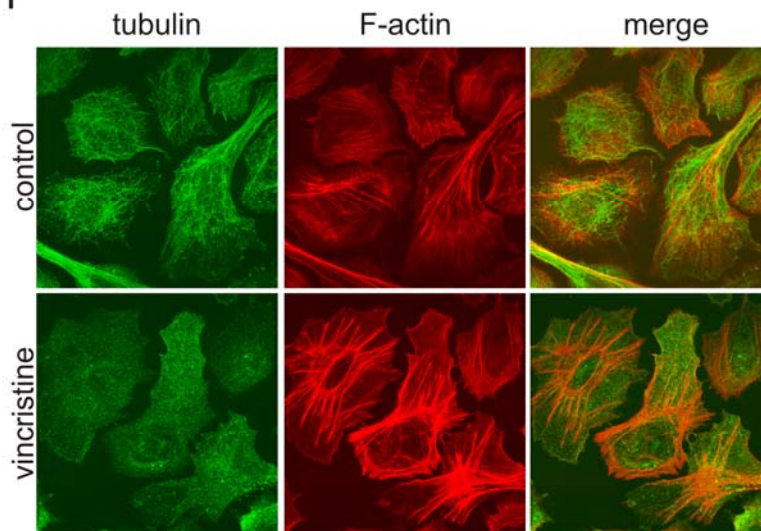


Fig.S2

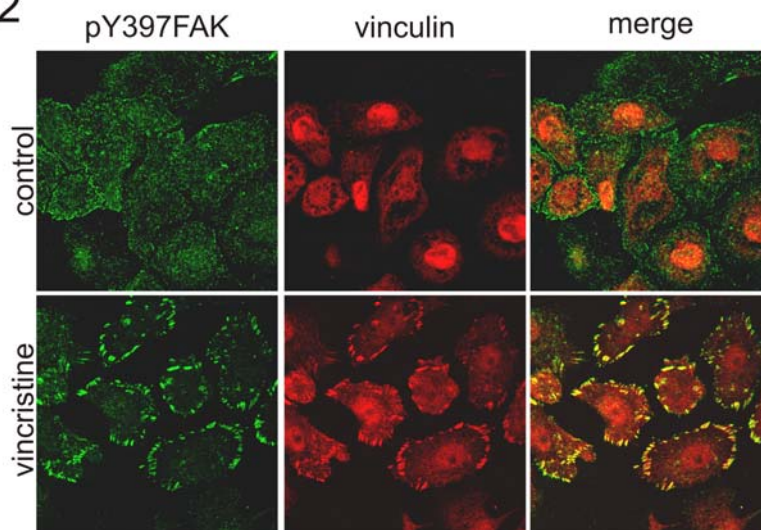


Figure. S1: Vincristine induces microtubule disruption and actin stress fiber formation. MTLn3 cells were treated with 50 nM vincristine for 8 hr. After fixation, cells were immunostained with the antibodies against tubulin (green) and actin (red).

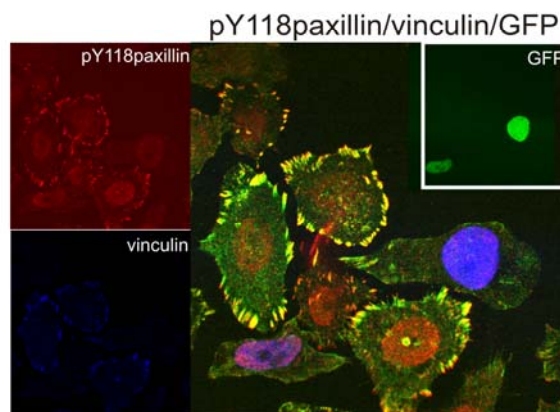
Figure. S2: Vincristine induces focal adhesion formation. MTLn3 cells were treated with 50 nM vincristine for 8 hr. After fixation, cells were immunostained with the antibodies against pY397 FAK (green) and vinculin (red).

Movie S3: Vincristine reduces the dynamics of focal adhesions. GFP-paxillin MTLn3 cells were treated with 50 nM vincristine for 8 hr and cells were visualized with total interference reflection fluorescence (TIRF) microscopy for 4 hr with 5 min/frame intervals. Movie S3A and S3B are representative movies (S3A for control cells (left DIC and right TIRF) and S3B for vincristine treated cells (left DIC and right TIRF)).

Figure. S4: JNK inhibition with DN-SEK rescues vincristine-induced focal adhesion formation. MTLn3 cells were transiently transfected overnight with GST-DN-SEK1 and GFP-Histon2B (ratio 20:1) and treated with vincristine (50 nM, 8 hr). Cells were fixed and stained with the antibodies against pY118paxillin (left top, red) and vinculin (left bottom, blue). Top right panel indicates the transfected cells that are GFP-H2B positive. (Note: due to software in the overlay cyan is GFP-H2B and green is vinculin).

movie.S3A and 3B

Fig.S4



CHAPTER 5

The serine178 residue of the focal adhesion-associated scaffold protein paxillin determines EGF-induced cell migration of metastatic breast tumor cell MTLn3

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ABSTRACT

The focal adhesion-associated scaffold protein paxillin plays a prominent role in cell migration. Recent data indicate JNK-mediated phosphorylation of paxillin at serine178. Here we investigated the role and mechanism of paxillin-Ser178 in the control of EGF-induced cell migration of the highly metastatic rat mammary carcinoma MTLn3 cells. EGF induced transient activation of JNK in MTLn3 cells, which was associated with JNK-dependent Ser178-paxillin phosphorylation. MTLn3 cells expressing a GFP-tagged Ser178Ala (S178A)-paxillin mutant had a phenotype with reduced proliferation, slower attachment and wound healing rate, compared to GFP-wt-paxillin expressing cells. Furthermore, the mutant cells did not undergo EGF-induced random cell migration sufficiently. EGF treatment resulted in limited phosphorylation at Ser178 of endogenous paxillin in S178A-paxillin MTLn3 cells. Moreover, EGF was unable to efficiently activate the PI3K/AKT and ERK pathways in these cells, compared to wt-paxillin cells. This suggests that Ser178-paxillin is important to control the EGF-induced signaling. Since paxillin phosphorylation at Ser178 is important to control cell migration of breast tumor cells, this phosphorylation event may also be important for breast tumor progression and metastasis formation.

INTRODUCTION

Breast cancer represents the most common worldwide type of cancer among women. The occurrence of distant metastases, or secondary tumors, is related to a very poor disease prognosis. The formation of secondary tumors involves distinct steps at the cellular level, including detachment, migration, invasion, extravasation and proliferation (1). To combat breast cancer metastasis more efficiently, improved insights into biological mechanisms in metastasis formation are of great importance. Focal adhesions, the closest contacts between cells and ECM, are important sites for signaling events (2-4). At focal adhesions, structural and enzymatic molecules act together to facilitate growth factor-stimulated and cell adhesion-dependent signaling, which are crucial in the different steps of the metastatic process (5,6).

Paxillin, a 68 kD multidomain adaptor protein, is associated with focal adhesions, where it functions as a scaffold to integrate multiple signaling pathways (4). At the N-terminus paxillin contains five leucine-rich LD domains (consensus LDXLLXXL) and several SH2 and SH3-binding domains; at the C-terminus paxillin contains four double zinc-finger LIM domains. LD domains facilitate the binding of a large array of binding partners, including integrin-linked kinase (ILK), actopaxin, G-protein coupled receptor kinase-interacting protein (GIT), focal adhesion kinase (FAK) and vinculin (7-10). The LIM domains of paxillin mediate the interaction with PTP-PEST and tubulin (11). LIM3, together with LIM2, targets paxillin to focal adhesions (11).

Throughout the paxillin molecule are many potential phosphorylation sites, including tyrosine, serine and threonine, which have all been mapped by mass spectrometry (12-14). Growth factor and integrin-mediated phosphorylation of paxillin at Tyr31 and Tyr118 induces the formation of a paxillin-Crk complex at focal adhesions and is essential for cell migration (15-17). Subsequently Crk-DOCK180 mediates the activation of Rac to enhance migration through lamellipodial extension (18-20). In NBT-II bladder tumor cells, induction of paxillin Tyr 31/118 phosphorylation and its association with CrkII are involved in cell adhesion, spreading and motility (20). Also Ephrin B1-stimulated cell migration requires phosphorylation of paxillin Tyr31/118 as well as the LD4 domain in a variety of cell types (21).

Serine/threonine phosphorylation of paxillin is observed by growth factor-mediated signaling, cellular stress and during mitosis (22-25). For example, adhesion stimulates the phosphorylation at Ser188/190 by an unknown kinase, as well as serine and threonine residues within LIM domains 2 and 3 (26). In addition, phosphorylation of paxillin at Ser273 has also been reported to regulate cell adhesion and protrusion dynamics via enhancing paxillin-GIT1 binding and promoting localization of a GIT1-PIX-PAK signaling module near the leading edge (12). Furthermore, p38 MAP kinase targets serine 85 in the process of neurite outgrowth (23). Finally, phosphorylation of paxillin at serine 178 is involved in EGF-stimulated cell migration via JNK (26,27) as well as in microtubule disruption condition which is also in association with JNK activation (see chapter 4). Many cell processes in tumor development depend on growth factor-mediated signaling, including EGF and HGF, which involves the activation of different MAPK family members. Therefore, it is important to further explore and understand the exact role of Ser178-paxillin phosphorylation by growth factors in cell migration and proliferation. Here we studied the role of Ser178-paxillin in the highly metastatic breast tumor cell line MTLn3.

We generated MTLn3 cell lines stably expressing either wt paxillin or Ser178Ala (S178A) mutant paxillin. S178A-paxillin significantly decreased cell proliferation and adhesion. Cell migration under control conditions (serum starvation) or after EGF stimulation was inhibited. S178A-paxillin suppressed JNK-mediated phosphorylation of endogenous paxillin under control and EGF conditions. The data indicate that Ser178 phosphorylation of paxillin after growth factor stimulation is essential to control cell migration and efficient activation of downstream signaling events including the PI3K/AKT and MEK/ERK signaling pathways. Altogether we conclude that the serine 178 residue of paxillin is an important player in cell proliferation and migration of metastatic breast tumor cells, and may be important for *in vivo* metastasis formation.

MATERIALS AND METHODS

Chemicals and Antibodies- Alpha modified minimal essential medium without ribonucleosides and deoxyribonucleosides (α -MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin and geneticin (G418 sulphate) were from Life Technologies. Rat tail collagen type I was from Upstate Biotechnology. LipofectAMINE Plus transfection reagents were from Invitrogen. Bradford protein assay was obtained from Bio-Rad and polyvinylidene difluoride (PVDF) membranes were from Millipore. Primary antibodies were anti-paxillin (BD), anti-tubulin, anti-GFP (Sigma, St. Louis, MO), anti-pT183/pY185-JNK (Promega), anti-Phospho-Thr202/Tyr204 ERK1/2, anti-pSer473-AKT (Cell signaling), anti-pSer178-paxillin (Abcam, Cambridge, UK). All secondary antibodies were from Jackson (GαRb-AP, GαM-HRP, GαRb-HRP or GαM-CY5). The Western-Star immunodetection system (Tropix kit) was from Applied Biosystems and ECL Plus reagent was from Amersham. Hoechst 33258 and rhodamine-phalloidin were from Molecular Probes and Aqua Poly/Mount was from Polysciences. All other chemicals were of analytical grade.

Cell Culture- MTLn3 cells were cultured as before (28). To generate stable cell lines, MTLn3 rat mammary carcinoma cells were transfected with GFP-paxillin or GFP-paxillinS178A along with empty vector pcDNA3 using LipofectAMINE plus reagents according to manufacturer's procedures. Stable transfectants were selected using G418 at a concentration of 500 μ g/ml. Individual clones were picked and maintained in α -MEM supplemented with 5% (v/v) FBS containing 100 μ g/ml G418 (complete medium). Clones were regularly analyzed for the expression of GFP constructs by flow cytometry analysis, western blotting and immunofluorescence. Cells were used for up to 8 passages and expression levels of GFP tagged proteins remained stable during experiment period. For stimuli experiments, 80-90% confluent cells were starved for 4hr and stimulated with EGF (10 nM) or HGF (5 ng/ml) for indicated time periods.

Proliferation, Attachment and Wound Healing Assay- For proliferation assay, cells were seeded in complete medium on 6 well-plates for 24, 48, 72 or 96 hrs. Cells were detached and the amount of cells was determined by counting. For cell attachment assay, cells were starved for 1 hr in serum-free medium and detached. Equal amounts of cells were plated in complete medium on collagen-coated 6-well plates. After 30, 60, 90 and 120 minutes, attached cells were trypsinized and counted. For wound healing assay, monolayer cells were scratched using a pipette tip to generate a wound. Plates were then washed with medium and wounds were photographed using phase contrast microscopy with a Nikon Coolpix digital camera. Plates were then incubated in α -MEM supplemented with 1% (v/v) FBS for 20 hrs. Wounds were photographed again and wound closure was determined using Image J software.

Live Cell Imaging- Cells were cultured in glass-bottom plates overnight and starved for 4 hrs followed by visualization with high throughput microscopy for 1 hr on a Nikon TE

2000-E microscope equipped with perfect focus system in a humid climate of 37°C and 5% CO₂. Subsequently, cells were treated with EGF or HGF and the exactly same fields were visualized for 1 hr. Movies were captured five minutes per frame with 20x objective. Cell speed was determined by tracking cell center and calculating the distance between two sequential frames. Cell dynamics were measured with cell surface area change between two sequent frames by homemade macro adopted in Image-Pro Plus (version 5.1, Media Cybernetics Inc., Silver Spring, MD).

TIRF and FRAP- Total internal reflection fluorescence (TIRF) microscopy was performed on GFP-paxillin MTLn3 cells and GFP-S178A-paxillin in a climate control chamber. TIRF movies were captured on a Nikon TIRF microscope system (Eclipse TE 2000-E, Nikon with automated stage) with framing every 5 minutes for 4 hrs using NIS-elements AR software (Nikon). To determine the turnover of GFP-tagged paxillin in individual focal adhesions, fluorescence recovery after photobleaching (FRAP) was performed as follows: photobleaching was applied to a small area covering a single focal adhesion for 1 s with laser intensity of 50 μ W. Redistribution of fluorescence was monitored with 100 ms time intervals at 7.5 μ W starting directly after the bleach pulse. Approximately 20 focal adhesions (each in distinct cells) were averaged to generate one FRAP curve for a single experiment. All measurements were performed at 37 °C using a heating stage with feedback temperature control and the experiment was performed on at least three different days. Images were analyzed with Image software (Zeiss). The relative fluorescence intensity of individual focal adhesion was calculated at each time interval as follows: $I_{rel}(t) = (FA_t / FA_0)$, where FA_t is the intensity of the focal adhesion at time point t after bleaching, FA_0 is the average intensity of the focal adhesion before bleaching. The fluorescent curves were analyzed with non-linear regression analysis (GraphPad Prism 5).

Gel Electrophoresis and Immunoblotting- Western blot analysis was performed as before (29). Cells were scraped in ice-cold TSE (10 mM Tris, 250 mM sucrose, 1 mM EGTA, pH 7.4) plus inhibitors (10 μ g/ml aprotinin, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride). After sonication, protein levels were determined using the Bradford protein assay with IgG as a standard. Equal amounts (25 μ g) of cellular lysates were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in either 0.2% (w/v) casein in TBS-Tween20 (for phospho-state specific antibodies) or 5% (w/v) BSA in TBS-T (for other primary antibodies) and probed with primary antibody overnight followed by sufficient washes and incubation with secondary antibodies. Alkaline phosphatase (AP)-conjugated secondary antibodies for phospho-proteins were detected with the Western-Star immunodetection system. For detection of horseradish peroxidase (HRP)-conjugated antibodies, ECL Plus reagent was used and followed by visualization on a Typhoon Imager 9400 (520nm, blue laser).

Immunofluorescence- Cells were seeded on collagen coated glass coverslips. Cells were briefly washed in PBS, followed by fixation in 3.7% formaldehyde for 10 min at room

temperature. After washing, coverslips were blocked in TBP (0.1% (w/v) Triton X-100, 0.5% (w/v) BSA in PBS, pH 7.4). Incubation with primary antibodies diluted in TBP containing 0.05% (w/v) NaN_3 was carried out overnight at 4°C. Primary antibodies were against paxillin. Coverslips were incubated with secondary antibodies conjugated to Cy5. After sufficient washing, coverslips were mounted on glass slides using Aqua Poly/Mount. Cells were visualized using a Bio-Rad Radiance 2100 MP confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted fluorescence microscope and a 60X Nikon objective.

Statistical Analysis- Student's t test was used to determine significant differences between two means ($p < 0.05$).

RESULTS

Mutation of Paxillin Serine178 Inhibits MTLn3 Cell Proliferation and Wound Healing Capability and Attenuates Cell Attachment- Paxillin is implicated in EGF-stimulated cell migration of rat bladder tumor epithelial cells (NBT-II) through JNK-mediated phosphorylation at paxillin Ser178 (27). Firstly we determined the involvement of JNK in EGF-induced migration of MTLn3 mammary adenocarcinoma cells. Exposure to EGF caused a rapid onset of random cell migration in MTLn3 cells, which was inhibited by an inhibitor of JNK, SP600125, indicating the requirement for JNK in cell migration (Fig. 1A). Next we determined the involvement of paxillin in this process. Treatment with EGF induced the transient phosphorylation of paxillin at Ser residue 178 in association with JNK activation. Importantly, this paxillin phosphorylation was dependent on JNK, since the inhibitor SP600125, prevented the phosphorylation at Ser178 (Fig. 1B). These data indicate that in MTLn3 cells EGF-induced phosphorylation of paxillin at Ser178 is mediated by JNK.

To further investigate the role of paxillin Ser178 in cell migration and proliferation, we generated MTLn3 cell lines stably expressing either GFP-tagged wt-paxillin or mutant GFP-paxillin in which the serine residue 178 was replaced by alanine (further referred to as S178A-paxillin). Clones of MTLn3 cells stably expressing GFP-wt-paxillin or GFP-S178A-paxillin were evaluated by flow cytometry (data not shown), western blotting and immunofluorescence (data not shown). Three wt-paxillin clones and three S178A-paxillin clones were selected for further experiments. Expression levels were equal in all three wt clones; one S178A clone had a lower expression level most likely due to reduced number of GFP-positive cells (Fig 2A). Expression levels of recombinant proteins remained stable for at least 8 passages (data not shown). Next, we determined the effect of S178A-paxillin expression on cell proliferation. S178A-paxillin expression significantly reduced the growth rate of MTLn3 cells (Fig.2B). This suggests that S178A-paxillin disturbs the essential cell proliferation signaling.

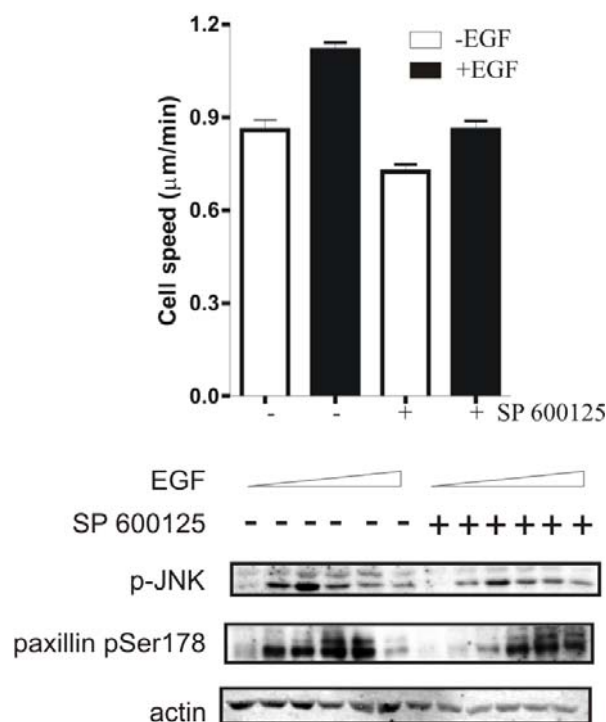


Figure 1: EGF signaling induces JNK-paxillin involved cell migration. MTLn3 cells grown in collagen-coated glass-bottom plate were pretreated with or without JNK inhibitor SP600125 for 30min and stimulated with EGF. Live cell migration was visualized on Nikon Eclipse TE2000-E PFS microscope (Nikon). Cell migration speed was analyzed as described in Materials and Methods section (A). Monolayer cells were pretreated with SP600125 for 30 min and followed with indicated incubation of EGF for 0, 5, 10, 20, 30, 60 min. Cell lysates were collected and separated with SDS-PAGE, and probed with pSer178-Paxillin and pJNK antibody (B).

Given the prominent role of paxillin in focal adhesion formation and dynamics, we next examined the effect of S178A-paxillin expression on MTLn3 cell attachment and spreading. For this purpose cells were seeded on collagen-coated dishes and allowed to attach for 30, 60 90 and 120 minutes. Significantly less S178A-paxillin cells, compared to wt-paxillin cells, attached. Importantly, while most of wt-paxillin cells had already spread, most of the S178A-paxillin cells remained round and presented a smaller surface area even when they attached and spread (Fig. 2C). This indicates that Ser178 of paxillin is essential for efficient attachment and spreading of MTLn3 cells to collagen. Next we determined the effect of S178A-paxillin on cell migration in an artificial wound healing assay. The closure speed of artificial wounds was determined after 20 hrs (Fig. 3). While wt-paxillin cells had closed the wound by 83 %, in sharp contrast, S178A-paxillin cells had only closed 25% of the wound. In conclusion, these above results indicate that S178A-paxillin affects different aspects of cell adhesion and migration.

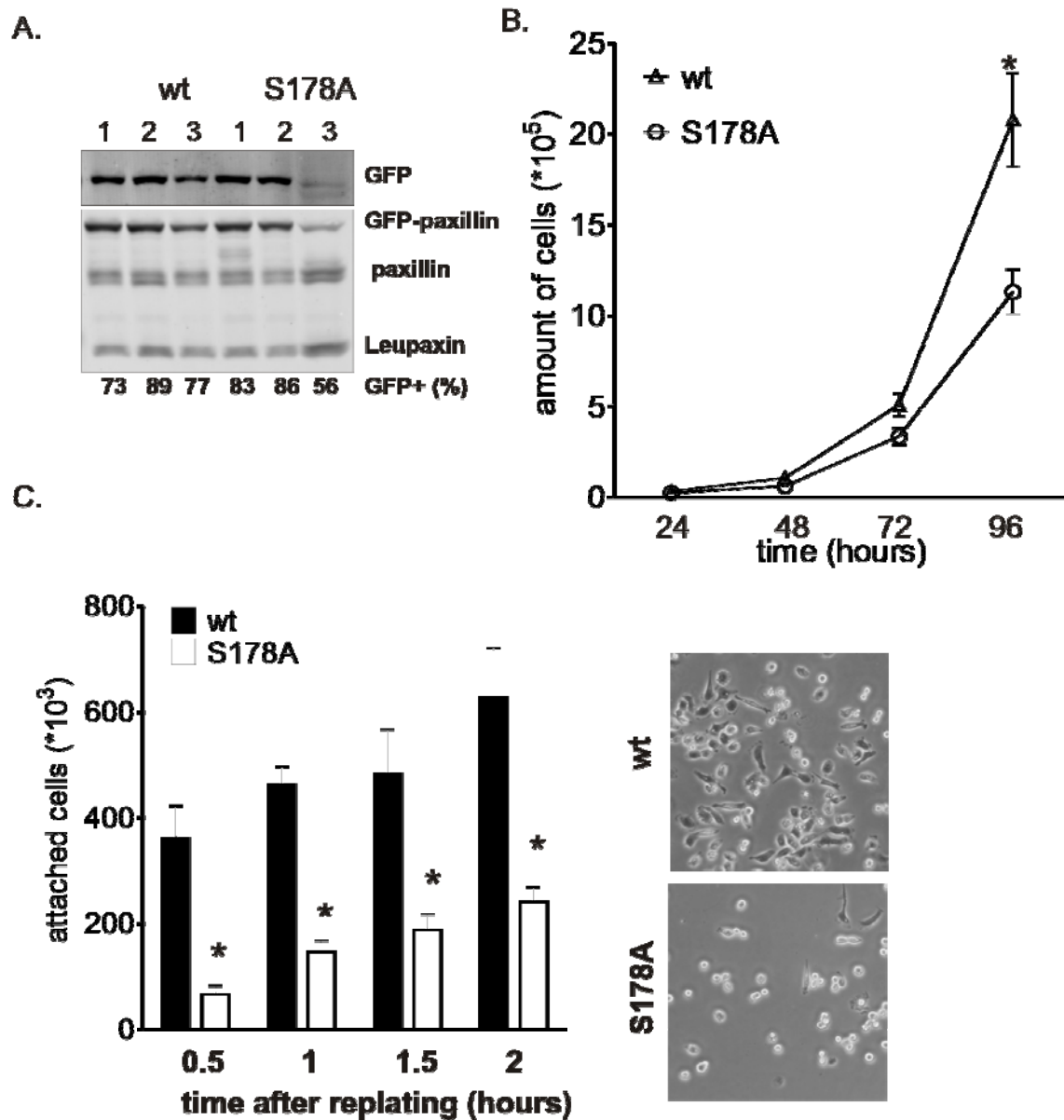


Figure 2: Ser178 residue of paxillin regulates cell proliferation and attachment. 3 different colonies for wt and S178A cells were picked for further research. Cell lysates for each cell line were collected and analyzed for GFP-paxillin expression by western blotting (A). Equal amounts for each clone (3 wt-paxillin clones and 3 S178A-paxillin clones) were cultured for indicated time periods and cell proliferation was determined by cell counting (B). Cell adhesion assays were performed as described in Materials and Methods section for all the wt and S178A clones. Note that S178A-paxillin cells are defective in efficient cell spreading (right panel) (C). Data shown are results from three independent experiments (mean \pm SD; $n=3$).

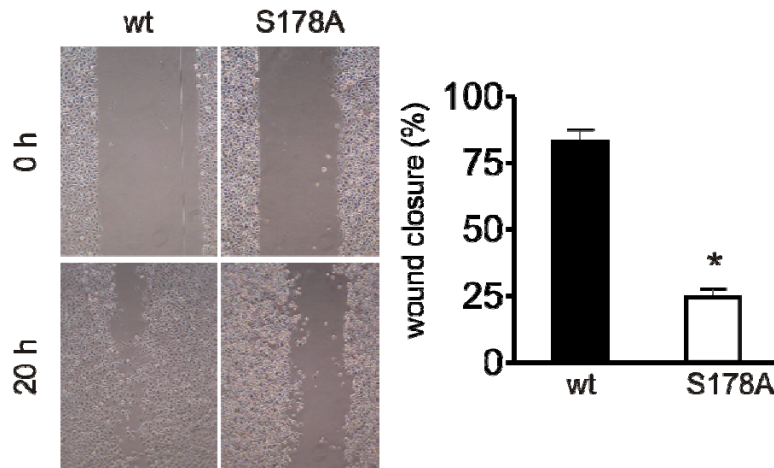


Figure 3. GFP-S178A-paxillin reduces wound closure rate.

The wound healing assay was performed as described in Materials and Methods. The wounds at indicated time points were photographed (Left). The length of wounds was measured in Image J and migration was expressed as the percentage of wound closure (Right).

Reduced Dynamics of GFP-S178A-paxillin at Focal Adhesions- To investigate the mechanism of the inhibitory effect of S178A-paxillin on cell migration, next we determined the dynamics of S178A-paxillin at focal adhesions. First, we evaluated the localization of GFP-wt-paxillin and GFP-S178A-paxillin in MTLn3 cells. With normal confocal microscopy, clear localization at focal adhesion was difficult to determine, although both GFP-wt-paxillin and GFP-S178A-paxillin were present at membrane ruffles and co-localized with paxillin in the same staining pattern indicating spatial functionality of both wt and mutant GFP paxillin constructs (Fig. 4A). To discern localization at focal adhesions we used TIRF microscopy, which allowed the detection of GFP signal in cells at the focal plane where cells make direct contact with the coverslip. TIRF microscopy indicated that both wt-paxillin and S178A-paxillin were localized at focal adhesions (Fig. 4B), thus indicating that the localization of S178A-paxillin at focal adhesions does not disturb the formation of focal adhesions. Next we investigated the dynamics of wt-paxillin and S178A-paxillin at focal adhesions. For this purpose we performed fluorescence recovery after photobleaching (FRAP) experiment with GFP-wt-paxillin and GFP-S178A-paxillin cells. Interestingly, GFP-S178A-paxillin cells showed the same recovery rate as GFP-wt-paxillin under control conditions. EGF stimulation increased the fluorescence recovery rate in GFP-wt-paxillin cells but not in GFP-S178A-paxillin cells (Fig. 4C). Thus, Ser178 at least in part determines the turnover of paxillin at focal adhesions and this residue mutant disturbs the cell response to EGF.

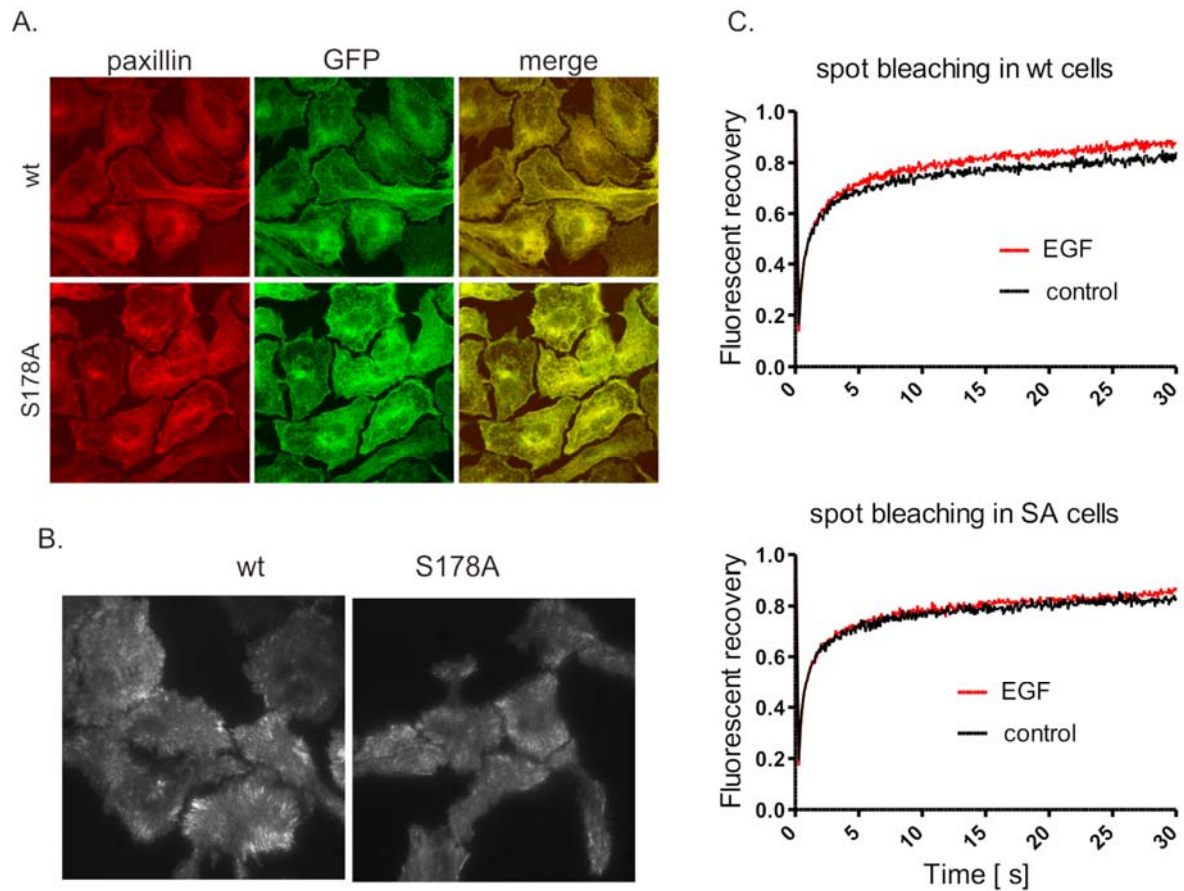


Figure 4: GFP-S178A-paxillin competitively co-localizes with endogenous paxillin at focal adhesion sites and decreases EGF-induced turnover of focal adhesions. GFP-wt-paxillin and mutant Ser178Ala cells were fixed for immunofluorescent assay and stained with mouse anti-paxillin antibody (A). A single frame from TIRF movies for GFP-wt-paxillin and mutant Ser178Ala cells (B). FRAP assay with GFP-wt-paxillin cells and mutant Ser178Ala-paxillin cells. Cells were treated with or without EGF for 20 mins before FRAP (C). Data shown are representative results from three independent experiments.

S178A-paxillin Inhibits Cell Motility in Serum Starved and EGF-Treated Conditions, but HGF Stimulates More Sufficiently- Next we investigated the effect of S178A on EGF-induced cell migration. Both wt-paxillin and S178A-paxillin cells were treated with EGF (10 nM) followed by the analysis of random cell migration. While wt-paxillin cells rapidly formed lamellipodia and became highly motile, this did not happen for S178A-paxillin cells (movie not shown). Since S178A-paxillin acts as a dominant negative construct in these cells, S178A-paxillin would compete for the localization of endogenous paxillin at focal adhesions and we reasoned that endogenous paxillin should lose either the basal phosphorylation level of Ser178 and/or the capability of being phosphorylated by JNK at Ser178. Indeed, EGF stimulation hardly activated phosphorylation of endogenous paxillin; as expected GFP-S178A-paxillin in these cells was not phosphorylated at all. In wt-paxillin cells, both endogenous and GFP-wt-paxillin were phosphorylated at Ser178 after EGF treatment (Fig. 6 top).

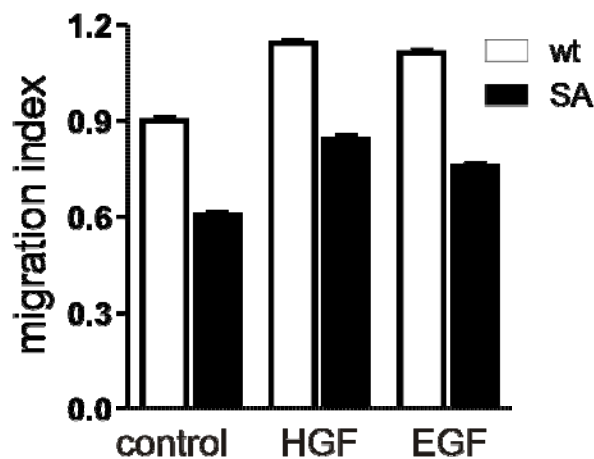


Figure 5: EGF and HGF induce cell migration and dynamic differently in GFP-wt-paxillin and mutant Ser178Ala cells. Random migration assay was done as mentioned in Materials and Methods.

Cell scattering is also induced by other growth factors, including hepatocyte growth factor/scatter factor (HGF) (30,31). HGF also induces activation of JNK in various cell types (32). We wondered whether HGF would be able to induce cell scatter in mutant cells. Treatment with HGF (5 ng/ml) induced cell migration in wt-paxillin cells. While S178A-paxillin did hardly migrate under serum starvation conditions, HGF stimulated cell migration better than EGF in S178A-paxillin cells (Fig. 5). These data indicate that, apparently, the cell migration machinery is functional in S178A-paxillin cells. Interestingly, in S178A-paxillin, HGF was capable of phosphorylating endogenous paxillin at Ser178 to a similar extent as in wt-paxillin cells; again S178A-paxillin was not phosphorylated after HGF treatment (Fig. 6 bottom). This suggests the phosphorylation of Ser178 is crucial for cell migration.

S178A-paxillin MTLn3 Cells have Reduced EGF-induced Activation of AKT and ERK-

Finally, we determined the possible mechanism by which S178A-paxillin affects EGF-induced cell migration. Since EGFR signaling is regulated and trans-activated at focal adhesions by both integrins (33,34) and FAK (35-37), we reasoned that possibly S178A-paxillin would disturb the downstream signaling of the EGFR. To investigate this, both wt-paxillin and S178A-paxillin cells were treated with EGF and downstream activation of both AKT and ERK was determined by western blotting. While EGF caused activation of AKT in wt-paxillin cells, proper AKT activation by EGF was inhibited in S178A-paxillin. Also ERK activation was slightly reduced in S178A-paxillin cells compared to wt-paxillin cells after EGF treatment, albeit less significant than that for AKT (Fig. 7 top). We then evaluated whether S178A-paxillin cells responded normally to HGF. Indeed, HGF treatment resulted in similar levels of phosphorylated ERK in both wt-paxillin and S178A-paxillin cells (Fig. 7 bottom). While the activation of AKT was reduced in S178A-paxillin cells, the overall activity of AKT by HGF stimulation was higher, compared to EGF stimulation (compare Fig. 7 top and bottom panels). These data suggest

that S178A-paxillin affects EGFR-mediated signaling and interferes with downstream signaling events that are essential to initiate and/or mediate cell migration.

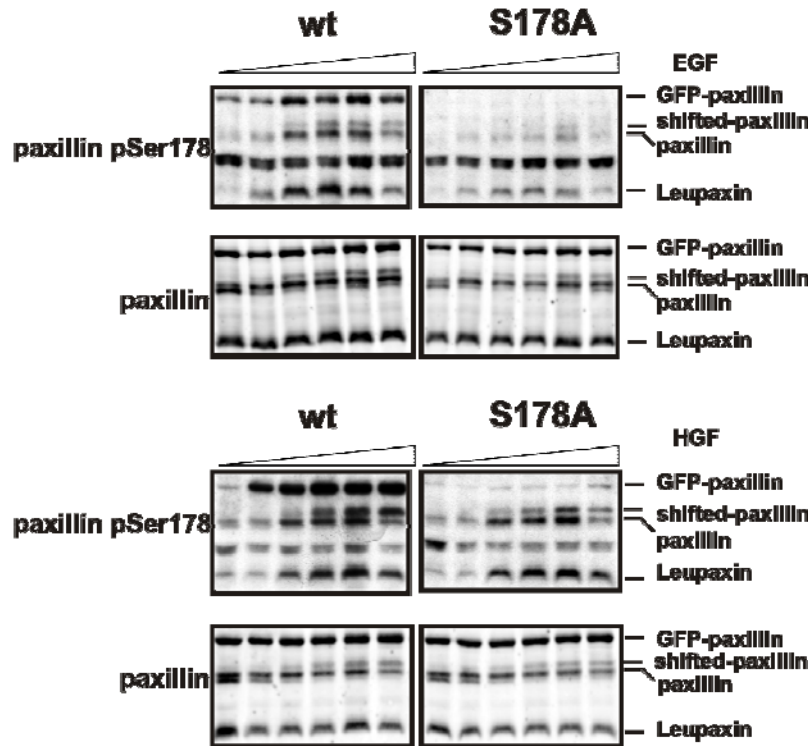


Figure 6: GFP-wt-paxillin and mutant Ser178Ala cells display different activation of signal pathway to EGF and HGF. 90% confluent cells were starved for 4hrs and stimulated with EGF (10nM, top panels) or HGF (5ng/ml, lower panels) for 0, 5, 10, 20, 30, 60 mins. Cell lysates were collected and western blot assay was performed to probe with antibodies against pSer178 paxillin and paxillin as mentioned in Materials and Methods.

DISCUSSION

The scaffold protein paxillin at focal adhesions regulates cell motility by regulating FA assembly and disassembly processes (3,17). In this study, we investigated the role of JNK-mediated phosphorylation of paxillin at Ser178 by generating cell lines stably expressing GFP-tagged paxillin in which the Ser178 residue was replaced by non-phosphorylatable alanine. Using these cell lines we were able to demonstrate that: 1) Ser178 phosphorylation is essential for efficient cell migration; 2) Ser178 phosphorylation determines the rate of cell proliferation; 3) Ser178 affects the efficiency of EGF-induced downstream signaling.

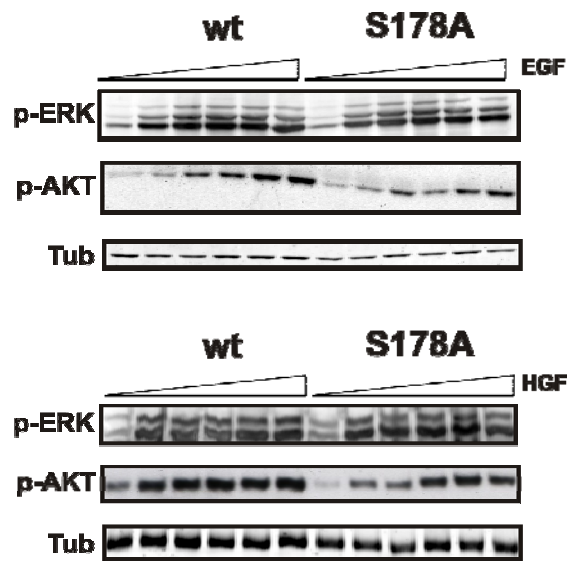


Figure 7: MTLn3 mutant Ser178Ala cells display a delayed and deficient activation of p-AKT compared to wt-paxillin cells in response to EGF. 90% confluent cells were starved for 4 hrs and stimulated with EGF (10 nM, top panels) or HGF (5 ng/ml, lower panels) for 0, 5, 10, 20, 30 or 60 min. Cell lysates were collected and analyzed by western blotting with antibodies against activated pERK and pAKT.

EGF is an important growth factor in the tumor metastasis process of MTLn3 cells (38). Our data indicate an important role for Ser178 of paxillin to control efficient EGF-induced signaling. We have performed Affymetrix cDNA microarray analysis of GFP-paxillin and GFP-S178A-paxillin (all clones depicted in Fig. 2). Interestingly, the preliminary data analysis indicate that the stable mutant S178A-paxillin cell lines express reduced levels of EGFR compared to wild type cell lines. Although further analysis of EGFR at the protein level is required, these data might explain both the reduced EGF-induced activation of AKT and ERK, in association with reduced EGF-induced cell migration. Since EGFR signaling is crucial for tumor progression, more *in vivo* work concerning the role of paxillin Ser178 and EGFR expression in tumor formation and progression should be carried out. In addition to EGFR, the levels of some cytoskeleton-associated proteins and matrix components were affected by S178A-paxillin expression, including secreted phosphoprotein 1 (fold change (mutant cells/wild type cells)=15), Rho GTPase activating protein 18 (FC=2), MMP3 (FC=6). These proteins may provide a clue for the cytoskeletal differences as well as the different capabilities in cell adhesion and migration observed in S178A-paxillin cells.

We have showed that JNK mediates the phosphorylation of paxillin Ser178 after EGF treatment. JNK is also activated by cellular stress conditions such as oxidative stress or microtubule disruption. As indicated in chapter 4, the microtubule disrupting agent vincristine induces a drastic and sustained activation of JNK in MTLn3 cells, which is associated with the modification of paxillin by phosphorylation of Ser178 as well as by an alternative modification of paxillin resulting in a mobility shift by SDS-PAGE. Apparently, the JNK activation by growth factors does not cause the same mobility shift of paxillin (see appendix figure at the end of this chapter). This suggests a more complex

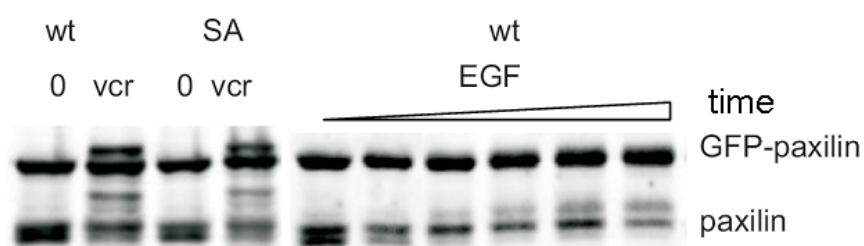
regulation of paxillin by JNK, which may be dependent on either the transient JNK activation after growth factor treatment, or alternative stress signaling pathways that are activated by stress conditions, such as microtubule disruption. Future work should establish the role of both of these paxillin phosphorylation events under in vivo conditions, and the relevance to both cancer progression and the sensitivity towards anticancer drug treatment, which typically involves the activation of JNK.

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Appendix

The comparison of paxillin modulation induced by vincristine (VCR) and EGF (shown is the immunoblot with paxillin antibody). Note the mobility shift of both GFP-tagged paxillin and endogenous paxillin by VCR but not by EGF.



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CHAPTER 6

DISCUSSIONS AND PROSPECTIVES:

The poor efficacy of breast cancer treatment is often a clinically intractable problem and the progressive stage of the disease is typically due to tumor metastasis formation. Primary tumors can be removed by surgery, chemotherapy and radiation therapies. However, small amount of remaining tumor cells in distant organs gain resistant capabilities and treatment of metastasis becomes more difficult. Therefore it is important to better understand the underlying metastasis process as well as the acquired drug resistance in breast cancer.

Chemokines and receptors in tumor microenvironment provide survival and chemotaxis signaling to tumor cells and are also involved in tumor growth and progression via leukocyte recruitment at tumor site. Focal adhesions (FAs) link cells to extracellular matrix and function as integration sites of signaling by extracellular stimuli (e.g. soluble factors, mechanical force, etc). Growth-factor-activation and integrin-clustering-induced FA-mediated signaling regulates cytoskeleton reorganization, cell adhesion, migration, proliferation and survival. The studies described in this thesis aimed to explore the role of FA signaling and chemokine receptor signaling in diverse cell biological processes relevant to metastasis and drug resistance (e.g. cell migration, proliferation and survival), in the context of breast cancer metastasis and treatment. In this thesis, we investigated chemokine and growth factor signaling (i.e. CXCR3 ligands and EGF) as well as anticancer drug-induced cellular stress response signaling (i.e. doxorubicin and vincristine) in cell migration and survival. Briefly, we described an overview of chemokine and chemokine receptor profiles in MTLn3 cells and established the relevance of an autocrine loop of CXCR3-ligands in cell migration. We further investigated the role and mechanism of FAs in a drug resistant phenotype of breast cancer cells. Finally, we described how JNK-mediated modification of paxillin was involved in microtubule disrupting agents-induced cellular stress response, as well as growth factor-induced cell migration process. In this chapter, I intend to integrate these findings and indicate the opportunities for future research.

Chemokine receptor signaling

In **chapter 2**, the expression profile of chemokines and receptors in MTLn3 cells is described. Although we only described CXC ligands and receptors, our microarray data indicated that other CC chemokines and receptors were also present (data not shown). Recently, it is noted that CXCR3 ligands CXCL9, 10 and 11 are potentially neutral antagonists of CCR3 and CCR5 which counteract the responses by such inflammatory chemokines (1) and both of these receptors are present in our MTLn3 cells (data not shown). Although we cannot exclude that these ligands may stimulate CCR3 and CCR5 in our model, knock down of CXCR3 with siRNA resulted in the reduced MTLn3 cell migration. This suggests that such an alternative activation by chemokine CXCL9, 10, and 11 is not biologically relevant *in vitro*. Aberrant expression of chemokines and receptors have often been found in diverse cancers, especially high expression of CXCR4 in breast cancers has been described and studied in detail. We found higher expression of

CXCR3 compared to CXCR4 in MTLn3, suggesting that CXCR3 might have stronger effect on tumor progression than CXCR4 in MTLn3. Moreover, the ligands of CXCR3 are expressed in these cells allowing direct and efficient autocrine activation, while the CXCR4 ligand CXCL12 is not expressed in MTLn3 cells.

We demonstrated the involvement of PI3K/AKT and MAPK/ERK in CXCR3-mediated cell motility and migration of MTLn3 cells. Signal transduction pathways derived from chemokine receptors are mainly investigated in the context of CXCR4 activation by CXCL12. The activation of downstream signaling events is mediated via G proteins, arrestin, PI3K, AKT, ERK and the transcription factors STAT and NF- κ B (2). Chemokine receptors undergo dimerization after binding to ligands followed by conformational changes. Consequently, heterotrimeric G proteins are activated and the dissociation of G $_{i\alpha}$ and G- β/γ from receptor triggers the traditional G-protein mediated pathways: the exchange of GTP to GDP on G α causes the activation of Src-Ras-ERK, and G $\beta\gamma$ triggers PI3K and PLC-PKC activation, followed by the formation of Cdc42-Rac-PAK complex and PKC-PYK2-FAK/Crk/p130Cas/paxillin complex. The latter complex is involved in actin cytoskeleton dynamics, cell polarity, cell adhesion and migration, and most likely, contributes to CXCR3 activation-mediated events (3-6). Another special pathway is related to the endocytosis and desensitization of chemokine receptor via receptor phosphorylation by G protein receptor kinases (GRKs) and binding of β -arrestin (7). Phosphorylation of the receptor by GRKs creates a binding site for arrestin and clathrin, resulting in receptor internalization and a shift to downstream cytoskeleton-related signaling. Arrestins also bind to microtubules, MAPK cascade components and non-receptor tyrosine kinase Src and Yes (8). All these connections are indicative for a tight association of chemokine receptor signaling with actin and microtubule cytoskeleton reorganization, cell dynamic/migration and survival/proliferation. More *in vitro* study to quantify actin dynamics and FA turnover during CXCR3-mediated cell migration in MTLn3 and other breast cancer cells need to be performed. Moreover, it will be important to further dissect signaling pathways downstream of CXCR3 and how these signaling pathways are relevant to *in vivo* tumor progression.

Considering the complexity of cancer types and cancer development, the large variety of chemokine/receptor, as well as the diversity of ligand-receptor binding, the exact mechanism and importance of chemokines and their receptors in cancer still remain largely unexplored. Future work should be focused on the role of CXCR3 and corresponding ligands in different *in vitro* and *in vivo* models. These may range from 3D cell culture models, zebra fish tumor implantation and metastasis models, and mouse tumor metastasis models, which have all been established in our lab. By using CXCR3-specific inhibitors or shRNA-silencing techniques we should elucidate the exact role of CXCR3 signaling in tumor and metastasis formation. Moreover, since chemokines act together with growth factors in tumor microenvironment, the crosstalk and synergy of downstream signaling between CXCRs and growth factor signaling would be important to study.

Focal adhesion signaling

Focal adhesions (FAs) contain more than hundred of components. These so-called integrin adhesomes form a complex network with 690 interactions (9). FAK and paxillin are intrinsic components of the adhesome and they each have 30 or more interactions with other kinases, adaptor proteins, phosphatases and cytoskeletal proteins, indicating the significance of FAK and paxillin at FA sites. In **chapter 3**, we studied FAK-mediated signaling using gene expression microarray and discovered genes and pathways that were altered by the expression of a dominant negative acting FAK splice variant, FRNK. Some of these identified pathways are involved in cell growth, cytoskeleton organization, cell shape and motility. In **chapter 4** and **5**, we mainly investigated the role of paxillin in cytoskeleton reorganization, cell migration and proliferation/survival in stress response and growth factor stimulation conditions. We established an interaction between paxillin and JNK in stress signaling caused by microtubule disrupting agents. In **chapter 5** the relationship between JNK and paxillin in tumor cell migration was established.

FAK and Fra-1 in focal adhesion dynamics, cell migration and metastasis formation

FAK and the transcription factor Fra-1 show increased expression in aggressive breast tumors than normal breast tissues (10, 11). In **chapter 3**, we have showed that FAK regulates Fra-1 expression, though it remains to be determined how FAK regulates Fra-1 expression. It has been established that Fra-1 levels are controlled by both ERK and AKT activity (12). FAK can downstream activate ERK (see **Introduction**), but we did not observe a differential activation of ERK after FRNK expression in our model by western blot staining (data not shown). However, FRNK did affect AKT activation under cellular stress conditions after doxorubicin treatment (13), indicating a dysregulation of proper FAK-AKT linkage. It remains unclear whether FRNK disturbs FAK-AKT linkage under normal condition and therefore is responsible for Fra-1 expression in MTLn3 cells. Both FAK and Fra-1 are implicated in MTLn3 cell migration ((14) and **chapter 3**). In other cell types, Fra-1 regulates cell motility and migration through inactivating β -integrin and keeping Rho activity low via ROCK/Rho kinase (15), with the exact regulatory mechanism still unclear. Also in MTLn3 cells, Fra-1 knock down interfered with FA turn over and increased FA size. We did not observe any Fra-1 accumulation at FAs in MTLn3 cells. Given the role of Fra-1 as a transcription factor, we propose that the effects of Fra-1 knock down on cell migration are rather related to its transcriptional activity. Fra-1 possibly regulates the expression of proteins that either are part of the adhesome or regulate the activity of adhesome components. Interestingly, in this context, by using a cDNA adenoviral library, FosB (another AP-1 transcription factor Fos family member) was identified in a 3D EMT-related morphogenesis screen (Price and van de Water, personal communication, our lab). This suggests the potential involvement of Fra-1 in 3D environment to modulate EMT process. Indeed, Fra-1 is highly expressed in mesenchymal breast tumor cell lines, but not in epithelial-like breast tumor cells (16). Possibly there is a relationship between Fra-1 expression/activity, actin cytoskeletal

network dynamics and FA turn over. More work should be done to define the exact role of Fra-1 in tumor cell survival and migration, and the consequences for metastasis formation and treatment *in vivo*.

There is clear link between MAPK pathway and AP-1 activity, including the regulation of Fra-1. Both EGF and phorbol-12-myristate-13-acetate (PMA) increase the expression of Fra-1 in MTLn3 (data not shown). Also doxorubicin treatment causes Fra-1 accumulation in MTLn3 cells (data not shown). Since CXCR3 and CXCR4 activate ERK and AKT signaling (see above and **chapter 2**), CXCR3 or CXCR4 activation may potentially also affect the expression of Fra-1, and thereby, tumor cell migration. We have not studied this aspect so far. In addition, Fra-1 is accumulated and hyper-phosphorylated in DNA-damage conditions via JNK and ERK (17). Based on the observations in **chapter 4**, we hypothesize that vincristine-induced activation of JNK in MTLn3 cells would potentially affect Fra-1 expression. This would raise the possibility that Fra-1 and/or other AP-1 components are involved in microtubule disruption-induced FA stabilization and cell contractility. So far, we have not investigated into it.

JNK signaling and focal adhesion regulation through paxillin

JNK is normally activated by growth factors (i.e. EGF and HGF) or cytokines (i.e. TNF α or interleukin-1). Also cell injury causes JNK activation and continuous cell stress induces sustained activation of JNK. Active JNK translocates to the nuclear and induces phosphorylation of c-Jun and activates AP-1. Phosphorylated JNK also localizes at FAs after growth factor exposure or cellular stresses, including treatment with microtubule disrupting agents such as vincristine. There are various JNK substrates (18) including transcription factors which are typically functional in the nucleus, such as c-Jun, scaffold proteins which regulate cell movement (e.g., paxillin and some other microtubule associated proteins), and proteins that regulate cell survival (e.g. Bcl-2 localized at the mitochondria and ER). In this thesis, we focus on JNK-mediated phosphorylation of paxillin at Ser178 in the context of vincristine-induced cytoskeleton reorganization and growth factor-induced cell migration, which were studied in **chapter 4** and **5** respectively.

In **chapter 4**, we have found that the JNK-paxillin axis is upstream of ROCK/MLC-dependent cell contractility induced by vincristine. JNK modulates LIM domain-containing proteins, including LIMK. LIMK-cofilin signal pathway is responsible for actin polymerization and stress fiber formation (19). Therefore, there is a possibility that vincristine-induced actin stress fiber formation is mediated through JNK-LIMK-cofilin-actin route. This needs further investigation. Although we mostly focused on JNK signaling, it is likely that many other kinases are involved in FA stabilization and cell contractility after microtubule disruption. Therefore, future emphasis should be placed on searching for other kinases involved in actin reorganization. Recently, a screening set up was developed in our lab to identify protein kinases and phosphatases that affect the formation of new FAs after treatment with another microtubule disrupting agent, nocodazole, as well as the FA turnover after washout of nocodazole. So far, the

preliminary screen identified several other MAPKases that are involved in this process. Probably it will provide a general overview of signal network that mediates actin/microtubule cytoskeleton dynamics and/or FA turnover. Further research will be performed to establish the role of these kinases in tumor cell migration and metastasis process and/or susceptibility towards anticancer drugs.

Breast cancer treatment

Molecular targeted anticancer therapy of breast cancer can be beneficial to the efficiency of traditional radiotherapy, chemotherapy and hormonal therapy. Given to the implication of chemokine receptors and specific FA proteins in tumor development and progression, they are potentially therapeutic targets to improve current traditional clinical treatment regimens. Some inhibitors for specific receptors and (non)receptor tyrosine kinases, such as ErbB1, ErbB2 and Src, have been developed for cancer treatment (19-22). These kinases are also directly involved in FA turnover and modulation of these kinases may indirectly affect FA downstream signaling. Alternatively, novel anticancer therapy can be developed to target chemokine receptors and kinases that are central in FA regulation. Modulation of FA downstream signaling will ultimately benefit breast cancer therapy. Here I will describe the current status and future perspectives of CXCR3 antagonists and FAK inhibitors.

CXCR3: a therapeutic target for breast cancer therapy?

In **chapter 2** we have showed that CXCR3 and CXCR4 are important in MTLn3 cell migration and invasion. Some antagonists and neutralizing antibodies for chemokine receptors have been developed for clinical trials by pharmaceutical companies, including CCR1, 2, 3, 5, CXCR1-4 (23). CXCR4 antagonist AMD3000 and other CXCR4 antagonists were tested in various tumor metastases and HIV. They prevent CXCL12-mediated cell chemotaxis, tumor formation and progression (24-26). A few CXCR3 antagonists were also studied in breast cancer *in vivo*. CXCR3 antagonist AMG487 was tested in a murine breast cancer lung metastasis model (27). The systemic blockade and tumor-specific inhibition of CXCR3 with AMG487 significantly prevented tumor metastasis, but had no effect on local tumor growth and survival. This was consistent with another study using tumor specific antisense nucleotides against CXCR3 (28-30). Interestingly, the anti-metastatic effect of AMG487 was lost in natural killer (NK) cell-depleted mice. This demonstrates that CXCR3-mediated tumor metastasis and formation require NK cells. Another selective CXCR3 antagonist NBI-74330 was tested *in vitro* and *in vivo* and it attenuates atherosclerotic plaque formation in LDL receptor-deficient mice (28, 31, 32). The effect of this inhibitor in cancer progression has never been investigated. Based on the above mentioned successful *in vivo* application, there is a great opportunity to compare effects of NBI-74330 and AMG487 and to substantiate the role of CXCR3 in breast cancer progression.

It is important to note that chemokines not only act on tumor cells to stimulate proliferation and migration, but also execute immunostimulatory effects and contribute to tumor repression or progression (33). The CXC subgroup of chemokines is divided into ELR+ and ELR- chemokines, based on the presence and absence of glutamic acid-leucine-arginine (ELR motif). The ELR+ chemokines are angiogenic and recruit tumor-associated neutrophils and macrophages which favor tumor progression via secreting matrix degrading enzymes and growth factors. In contrast, the ELR- chemokines, like CXCL9, 10, 11, are angiostatic and recruit T lymphocytes and NK cells which are cytotoxic, and thereby suppress tumor development (34). However, the expression of CXCR3 in melanoma, colon and breast carcinoma seems to facilitate tumor metastasis formation to lymph nodes (28, 30, 35), suggesting that, like CXCR4, CXCR3 might have a special role of homing CXCR3-positive tumor cells to sites where IFN- γ -inducible chemokines, like CXCL9, 10, 11, are abundant. Since CXCR3 and its ligands have opposing roles in tumor development, a caution is required when CXCR3 antagonists are used as potential therapeutic targets and this might explain present failure of clinical trials (27). CXCR3 antagonists or chemokine CXCL9, 10, 11 neutralizing antibodies could be used as potential drugs to block the trafficking or self-survival and motile signaling in tumor cells. Future work should establish the role of chemokine CXCL9, 10, 11 and receptor CXCR3 in tumor metastasis in NK-depleted Fisher 334 rat and immune-deficient mice by using shRNA approaches to knock down receptor and ligands.

FAK as an anticancer therapeutic target

In **chapter 3** we have demonstrated that FAK is essential in regulating the efficacy of doxorubicin in either primary breast tumors or lung metastases by using conditional FRNK expression. Over expression of FRNK sensitizes different tumor cell lines against anticancer drugs such as 5-fluoroucil, doxorubicin, vincristine (36). This supports FAK as a potential anticancer drug target. The modulation of FAK *in vivo* could be achieved by siRNA, specific inhibitors, or extrinsic introduction of dominant-negative mutants such as FRNK (37-39).

FAK short interfering RNA (siRNA) shows high efficacy to downregulate FAK expression *in vitro* (40). Also, knock down of FAK in **chapter 3** clearly deleted FAK protein expression. Inhibition of FAK with short hairpin RNAs (shRNAs) prevents FAK function in cell adhesion, migration and proliferation in mouse breast cancer cell line 4T1 *in vitro* and suppresses tumor growth in heterotopic/orthotopic mice models *in vivo* (37). However, it may be difficult to deliver siRNA *in vivo* efficiently to tumor tissue because of rapid degradation of siRNA. Chemical approaches to modify siRNA stability as well as uptake in tumor cells can increase the efficacy of siRNA approaches to modulate FAK levels. So far, a modified polyethylenimine (PEI) gene carrier (41) and a neutral lipid liposome, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) (42) have been reported to introduce FAK siRNA successfully *in vivo*, which could be potentially utilized in breast cancer treatment and even modulated for breast tumor-selective target in the future.

So far, several FAK selective or dual inhibitors including PF562271 and TAE226, have been tested in (pre)-clinical studies of different cancer types (43, 44). Disturbance of FAK activity and function have a synergistic effect with some traditional chemotherapeutics, as we demonstrated for FRNK expression. PF562271 has a dual effect on FAK and Pyk2 with an IC_{50} in nano-molar scale *in vitro*. *In vivo*, maximal inhibition of FAK phosphorylation (78%) is obtained 1 hour after p.o. administration at 33 mg/kg dose in tumor-bearing mice and the inhibition (>50% inhibition of FAK phosphorylation) lasts above 4 hours with a single dose (45). Furthermore, dose-dependent tumor growth inhibition and regression were observed in a broad range of human s.c. xenograft models, including prostate, breast, pancreatic, colon, lung and glioblastoma, with no observation of weight loss, morbidity or death (45). The inhibitory mechanisms of PF562271 *in vivo* rely on anoikis/apoptosis and reduction of micro-vascular density (45). TAE226 has been tested in other tumor models, for instance, glioma, ovarian and esophageal carcinoma (46-48). TAE226 inhibits phosphorylation of FAK and downstream signaling effectors AKT and ERK. It decreases cell proliferation, adhesion, migration and invasion in glioma cells (48). It also has significant action in ovarian carcinoma by inhibiting FAK phosphorylation at Y397 and pY861, as well as cell growth in a time and dose-dependent way. Moreover, it shows a synergistic effect on docetaxel-mediated cell growth inhibition, tumor burden reduction and prolonged survival in tumor-bearing mice (47). All these studies indicate the prospective application of FAK inhibitors in therapy combination. Future work will investigate whether the effect on tumor growth is dependent on intrinsic mechanisms of tumor cells or through anti-angiogenesis by inhibiting vascular endothelial cell migration. Moreover, it would be interesting to determine the combined effect of these inhibitors and traditional anticancer drug in breast cancer metastasis models in our lab. Finally, it will be essential to define the specificity of these kinase inhibitors.

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CHAPTER 7

ENGLISH SUMMARY

NEDERLANDSE SAMENVATTING

ENGLISH SUMMARY

Breast cancer is the most common malignancy among women in the western world. The difficulties to treat breast cancer relate to breast cancer metastasis. Modulation of focal adhesion-mediated signaling can improve tumor resistance and benefit the therapeutic efficiency. This thesis focuses on focal adhesion signaling in various cell processes in breast cancer development. The long term goal is to discover an alternative way for breast tumor treatment through modulation of focal adhesion signaling.

In the **chapter 1**, I introduce the different signaling pathways implicated in cell survival, proliferation, migration and point out a few effect factors which are involved in tumor development and might be therapeutic targets for tumor treatment. I mainly focus on the importance of chemokines and growth factor (receptors), MAPKase pathways, the transcription factor AP-1 and the PI3K/AKT signaling pathway in breast cancer. Moreover, I put emphasis on focal adhesion-related protein FAK and paxillin that are essential in focal adhesion turnover and signaling. These proteins are central in the rest of the thesis.

Chemokines and receptors are important for regulation of tumor cell proliferation, migration and metastasis as well as the cellular compositions of the tumor microenvironment. Various chemokines and their receptors are expressed in different breast tumor stages and related to breast cancer progression. The roles of many receptor-ligand pairs in this process remain unclear. In **chapter 2**, the role of chemokine receptor CXCR3 in cell motility and migration was examined in the rat mammary carcinoma cell line MTLn3. Firstly the expression profiling of chemokines CXCLs and chemokine receptor CXCRs were performed by microarray and validated with RT-PCR. CXCR3 and its ligands CXCL10 and CXCL11 were prominently expressed in MTLn3 cells compared with CXCR4. Secondly, the activation of CXCR3 and CXCR4 were measured with calcium influx assay to validate the functionality. CXCL11 enhanced random cell migration and stimulated chemotaxis-driven cell invasion. The activation of CXCR3 was associated with the activation of MAPKase/ERK and PI3K/AKT. Pharmacological inhibition of an upstream activator of ERK, MEK, with the inhibitor U0126, or phosphoinositide-3 kinase with LY294002, inhibited the CXCL11-induced MTLn3 cell invasion. The disruption of autocrine loop of CXCR3-respective ligands impaired cell migration associated with reduced protrusion formation. Together, these data suggests that autocrine CXCR3 activation is important for MTLn3 tumor cell migration and invasion and a potentially important drug target for breast cancer treatment.

FAK, the central player at FA sites, is involved in cell survival and migration. In **chapter 3**, the FAK-mediated survival signaling was studied in rat breast cancer model in relation to the anticancer drug doxorubicin treatment. Conditional introduction of a dominant-negative acting FAK splice variant, FAK-related non kinase (FRNK), competes for the localization of endogenous FAK at focal adhesions, and, thereby, disturbs FAK-mediated

survival and migratory signaling at focal adhesions. FRNK expression does not significantly have inhibitory effect on cell proliferation or causes apoptosis in normal culture condition, however it sensitizes cell to doxorubicin treatment and inhibits cell spreading and migration to an artificial wound. Earlier work from our laboratory indicated that conditional FRNK expression inhibited primary breast cancer formation as well as the early steps of metastasis formation. FRNK expression did not inhibit outgrowth of macrometastasis. This allowed us to investigate the consequences of FRNK expression on the treatment with doxorubicin when metastases were already formed. FRNK expression sensitized both orthotopic MTLn3 cell breast tumors and experimental MTLn3 cell lung metastasis to doxorubicin. Gene expression profile and qRT-PCR indicated FRNK-expressing cells expressed less Fra-1 than non-expressing cells. Lastly, we validated the reduction of Fra-1 expression by FAK knock down with siRNA, as well. Fra-1 KD increased the focal adhesion formation and decreased the rate of its turnover. Moreover, Fra-1 KD sensitized MTLn3 cells to doxorubicin. Finally, overexpression of Fra-1 rescued doxorubicin-induced cell apoptosis in FRNK expressing cells. Our study provides a link between tyrosine kinase FAK and transcription factor Fra-1 whereby FAK signaling supports Fra-1 expression.

FAs contain a variety of signaling molecules and adaptor proteins. We further investigated the focal adhesion associated adaptor protein paxillin in chapter 4 and 5. Paxillin regulates FA dynamic and it is involved in cell survival/proliferation and migration. In **chapter 4** we investigated the mechanism by which paxillin regulated the cell morphological changes and F-actin cytoskeletal reorganization after microtubule cytoskeleton disruption by anticancer drugs vincristine. The microtubule disruption agent vincristine induced cell cycle arrest, contractile phenotype with bigger focal adhesions in association with F-actin stress fiber formation. This was accompanied with the activation of MAPK/JNK pathway before the onset of apoptosis, and active JNK localized at focal adhesion sites. Paxillin was modified to a much lower mobility on PAGE. Moreover, paxillin was phosphorylated at Ser178. Both modifications of paxillin after vincristine treatment were dependent on JNK activity since an inhibitor of JNK, SP600125 prevented these. Also, SP600125 inhibited cell rounding and cell contractility. siRNA-mediated knock down of paxillin itself did not affect cell apoptosis, but inhibited vincristine-induced cell contractility and focal adhesion formation. The cell contractility was also inhibited by an inhibitor of ROCK, but this did not affect JNK activation and paxillin modification induced by vincristine treatment. Therefore, this study suggested a tight relationship between the vincristine-induced JNK activation, scaffold protein paxillin modification and regulation of focal adhesion dynamics and cell contractility.

Paxillin has a crucial role in cell migration and proliferation. Different growth factors, including EGF and HGF, activate JNK. JNK can phosphorylate paxillin on Ser178. We studied the role of paxillin Ser178 residue in growth factor mediated cell migration of MTLn3 breast tumor cells in **chapter 5**. Firstly, EGF induced Ser178 phosphorylation in MTLn3 cells in a JNK-dependent manner. Moreover, EGF-induced cell migration was

inhibited by SP600125, a specific inhibitor of JNK. The GFP-tagged Ser178Ala mutant of paxillin was expressed in MTLn3 cells; GFP-tagged wt-paxillin served as control. GFP-S178A paxillin inhibited MTLn3 cell attachment, cell migration and affected cell proliferation. Interestingly, the EGF-induced activation of PI3K/AKT and ERK signaling was reduced in S178A-paxillin cells compared to wt-paxillin cells. Thus, we propose that Ser178 of paxillin is important in the regulation of EGFR signaling, and thereby, may affect downstream signaling that typically facilitate cell migration and proliferation. Future studies should focus on the role of paxillin Ser178 in breast cancer growth, progression and metastasis formation *in vivo*.

In **chapter 6**, I further discussed the results and findings in the previous research chapters in the context of the literature and the present work in our lab. I indicated the potential therapeutic targets, ongoing therapeutic opportunities and also pointed out the prospective studies.

NEDERLANDSE SAMENVATTING

Borstkanker is een van de meest voorkomende kanker bij vrouwen in de Westerse wereld. Het probleem in de behandeling borstkanker is vooral het voorkomen en bestrijden van uitzaaiingen (metastasen). Remming van de signaal transductie via focal adhesions kan de tumor resistentie verminderen en is daarom een veelbelovende manier om uitzaaiingen te voorkomen. Dit proefschrift behandelt de rol van focal adhesie signalering in verschillende cellulaire processen die belangrijk zijn voor metastasering van borstkanker. Het uiteindelijke doel is om een geschikte behandeling van borstkanker metastasering te ontdekken/ontwikkelen die gebaseerd is op het moduleren van focal adhesie signalering.

In **hoofdstuk 1** wordt een overzicht gegeven van de verschillende signaleringsroutes die een rol spelen bij cel overleving, proliferatie en migratie. Er worden tevens een aantal factoren uitgelicht die betrokken zijn bij tumor ontwikkeling en mogelijk interessant zijn als therapeutische target voor de behandeling van borstkanker. De focus ligt op de rol van chemokines en groeifactoren, MAPKase routes en de transcriptiefactor AP-1 alsook de PI3K/AKT signaleringsroutes in borstkanker. De focal adhesie-gerelateerde eiwitten FAK en paxillin, twee eiwitten die essentieel zijn voor focal adhesie turnover en signalering en centraal staan in dit proefschrift, worden uitvoerig behandeld.

Chemokines en de corresponderende receptoren zijn belangrijk voor de regulering van tumorcel proliferatie en migratie en cellulaire samenstelling van de tumor micro-omgeving alsook de vorming van metastasen. Verschillende chemokines en hun receptoren zijn geïmpliceerd in verschillende stadia van borstkanker. De expressie van sommige chemokines correleert met de progressie van borstkanker. De rol van verschillende receptor–ligand-paren zijn in dit proces nog niet volledig ontrafeld. In **hoofdstuk 2** wordt de rol van de chemokine receptor CXCR3 in cel migratie in de borstkanker cellijn MTLn3 onderzocht. Allereerst werd het expressieprofiel van verschillende CXCL chemokines en de corresponderende CXCR chemokine receptoren bepaald met behulp van microarrays als ook RT-PCR. Voornamelijk CXCR3 en de liganden CXCL10 and CXCL11 kwamen tot expressie in MTLn3 cellen. Vervolgens werd de activering van CXCR3 en CXCR4 met behulp van vrij calcium-influx analyse gemeten om de functionaliteit van deze receptoren in MTLn3 cellen te evalueren; beide receptoren zijn actief in MTLn3 cellen. Blootstelling van MTLn3 cellen aan CXCL11 verhoogde de cel migratie en stimuleerde het chemotaxische-invasieve gedrag van deze tumorcellen. De activering van CXCR3 ging gepaard met de activering van MEK/ERK en PI3K/AKT signaal transductie routes. Farmacologische remming van een activator kinase van ERK, MEK, met de chemische remmer U0126, of remming van phosphoinositide-3 kinase (PI3K) met de remmer LY294002, verlaagde de CXCL11-geïnduceerde MTLn3 celinvasiviteit significant. Downregulatie van de CXCR3 receptor met RNA-interferentie middels siRNA transfecties remde de celmigratie veroorzaakt door blootstelling aan CXCR3 liganden. Samenvattend suggereren deze data dat de autocriene CXCR3 activering zeer belangrijk is voor MTLn3 tumor celmigratie en –invasie.

Blokkeren van de CXCR3 receptor zou daarom een potentieel belangrijke geneesmiddel target voor de behandeling van borstkanker progressie kunnen zijn.

FAK speelt een centrale rol in de regulering van de dynamiek van focal adhesions. Tevens is FAK betrokken bij de signaal transductie vanuit focal adhesions om processen als cel overleving en cel migratie te ondersteunen. In **hoofdstuk 3** wordt onze studie naar FAK-gemedieerde cel overleving in relatie tot antikanker therapie behandeld. Conditionele expressie van een dominant-negatieve variant van FAK, de FAK-gerelateerde non kinase (FRNK), competeert voor de lokalisatie van endogeen FAK op focal adhesions. Het gevolg hiervan is dat de FAK-gemedieerde cel overleving en cel migratie signalering wordt verstoord. FRNK expressie heeft geen remmend effect op cel proliferatie en leidt niet tot apoptosise onder normale celweek condities. Echter, FRNK expressie zorgt wel voor toename in gevoeligheid voor antikanker middelen zoals doxorubicin. In eerder onderzoek hebben we aangetoond dat conditionele expressie van FRNK zorgt voor een remming in de vorming van metastases; FRNK had hierbij geen effect op de uitgroei van macro-metastases. Dit gaf ons de mogelijkheid om het effect van FRNK-expressie op de behandeling met doxorubicine te bestuderen nadat de metastases al gevormd waren. Expressie van FRNK zorgde voor een toename in gevoeligheid van MTLn3 borsttumoren en experimentele MTLn3 metastases voor behandeling met doxorubicin. Het gen expressieprofiel en de qRT-PCR data lieten zien dat cellen die FRNK tot expressie brachten minder Fra-1 mRNA tot expressie brachten dan FRNK-negatieve cellen. Hetzelfde werd gevonden na remming van FAK doormiddel van een siRNA-gemedieerde knock-down van FAK. Omgekeerd leidde een knock-down van Fra-1 niet tot een veranderde FAK expressie, maar wel tot een toename in de vorming en grootte van focal adhesions. Belangrijk was het feit dat een Fra-1 knock down in de MTLn3 cellen deze cellen gevoeliger maakte voor doxorubicin. Daarentegen maakte overexpressie van Fra-1 MTLn3 cellen die FRNK tot expressie brachten juist weer ongevoelig voor doxorubicin-geïnduceerde celdood. Onze studie toont aan dat er een relatie is tussen de signaal transductie gemedieerd door FAK en de expressie van de transcriptie factor Fra-1. Fra-1 op zijn beurt reguleert weer de gevoeligheid van cellen voor celdood.

Focal adhesions bestaan uit verschillende signaleringsmoleculen en adapter proteïnen. In hoofdstuk 4 en 5 wordt het focal adhesion eiwit paxillin verder bestudeerd. Paxillin reguleert de dynamiek van focal adhesions en daarmee de migratie van cellen; daarnaast is het ook van belang in de signaal transductie van cel overleving. In **hoofdstuk 4** werd het mechanisme onderzocht hoe paxillin de morfologische veranderingen van de cel en de F-actin cytoskelet reorganisatie reguleert na beschadiging van het microtubuli cytoskelet na blootstelling met het cytostaticum vincristine. Zoals verwacht veroorzaakte vincristine een blokkade in de celcyclus. Daarnaast zorgde vincristine voor een verhoogde contractiliteit van de cellen gevolgd door opronding van de cellen. Dit ging gepaard met grotere focal adhesions en vorming van zogenaamde F-actin cytoskelet stress fibers. Onder deze condities vond ook een activering plaats van de MAPK/JNK route waarbij

actief JNK geassocieerd was met focal adhesions. Paxillin werd gefosforyleerd op serine residu 178 (Ser178), hetgeen voorkomen kon worden door een remmer van JNK (SP600125). SP600125 remde ook de contractiliteit en opronding van de cellen. Knock down van paxillin d.m.v. siRNA leidde niet tot apoptose, maar inhibeerde vincristine-geïnduceerde cel contractiliteit en de vorming van focal adhesions. Contractiliteit van de cellen werd ook geremd door een inhibitor van ROCK, maar deze beïnvloedde niet de activiteit van JNK alsook de phosphorylering van paxillin. Deze data suggereren een sterke samenhang tussen de JNK-activatie door vincristine, modificatie van het eiwit paxillin en de regulatie van focal adhesie dynamiek en cel contractiliteit. Deze gebeurtenissen kunnen van belang zijn voor het beter begrijpen van de mechanismen van werking van vincristine en suggereren dat paxillin of andere eiwitten betrokken bij de signaal transductie downstream van paxillin, een target kunnen zijn voor de behandeling van kanker.

Zoals hierboven al genoemd speelt paxillin een cruciale rol in cel migratie en proliferatie. Verschillende groeifactoren, waaronder epidermaal groeifactor (EGF) en hepatocyte groeifactor (HGF), activeren JNK. JNK kan vervolgens paxillin fosforyleren op Ser178. In hoofdstuk 5 hebben we de rol van het paxillin Ser178 residu in groeifactor gemedieerde celmigratie van MTLn3 borst tumorcellen bestudeerd. EGF induceerde de fosforylering van paxillin Ser178 in MTLn3 cellen hetgeen afhankelijk was van de activiteit van JNK. De door EGF geïnduceerde cel migratie was ook afhankelijk van JNK activiteit, want een specifieke remmer van JNK (SP600125) voorkwam deze cel migratie. Vervolgens hebben we MTLn3 cellen gemaakt die een variant van paxillin tot expressie brachten die niet meer gefosforyleerd kan worden op Ser178: GFP-Ser178Ala-paxillin; GFP-gekoppelde wt-paxillin werd gebruikt voor het genereren van de controle cellijnen. Expressie van GFP-S178A-paxillin remde de hechting van MTLn3 cellen aan de extracellulaire matrix; tevens was de migratie van de MTLn3 cellen geremd. Normaal leidt activatie van de EGF-receptor tot de activering van de PI3K/AKT en MEK/ERK signaal transductie route. Expressie van GFP-S178A-paxillin, maar niet GFP-wt-paxillin, verlaagde deze door EGF blootstelling geïnduceerde activatie van PI3K/AKT en ERK signalering. We gaan ervan uit dat serine residu 178 van paxillin belangrijk is voor de regulatie van de intracellulaire signaal transductie na activatie van de EGFR receptor en dat via deze weg paxillin betrokken is bij de regulatie van EGF-geïnduceerde cel migratie en proliferatie. In vervolg onderzoek zou de nadruk moeten liggen op het bestuderen van de rol van de fosforylering van paxillin op Ser178 door JNK in ontwikkeling en progressie van borstkanker.

In **hoofdstuk 6** worden de resultaten en bevindingen van dit proefschrift in de context van recente literatuur en resultaten van onze onderzoeksgroep bediscussieerd. Daarbij komen de potentiële therapeutische targets en huidige therapeutische mogelijkheden aan bod en wordt visie voor toekomstig onderzoek besproken.

List of abbreviations

α -MEM	alpha-modified minimal essential
AKT	protein kinase B (also abbreviated PKB)
AMC	7-amino-4-methylcoumarin
AP-1	activator protein 1
ARF	ADP-ribosylation factor, a small GTP-binding protein
ARP2/3	actin-related proteins2/3
ASAP1	the Arf-GTPase-activating protein 1
ATF	activating transcription factor
BSA	bovin serum albumin
CC (L/R)	C chemokine (ligand/receptor)
Cdc42	cell division cycle 42 ,a GTP binding protein
CDK	cyclin-dependent kinases
Chk	checkpoint kinases
c-MET	mesenchymal-epithelial transition factor
CXC(L/R)	CXC chemokine (ligand/receptor)
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF(R)	epidermal growth factor (receptor)
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FA	focal adhesion
FACS	fluorescence-activated cell sorting
F-actin	filament-actin
FAK	focal adhesion kinase
FAT	focal adhesion targeting domain
FERM	band 4.1, ezrin, radixin and moesin homology domain
Fra-1(fosl-1)	fos related antigen-1(fos like-1)
FRAP	fluorescence recovery after photobleaching
GAP	GTPase activating proteins
GFP	green fluorescent protein

GIT	G-protein-coupled receptor kinase interacting protein
Grb2	growth factor receptor-bound protein 2
GRAF	GTPase activating protein for Rho associated with FAK
GRK	G-protein receptor kinase
GST	glutathione-S-transferase
GTP	guanosine Triphosphate
HE	haematoxylin and eosin
HGF	hepatocyte growth factor
ILK	integrin linked kinase
I.P.	Intraperitoneal injection
JNK	c-Jun N-terminal kinase
KD	knock down
KO	knock out
LD	leucine-rich sequences
LIMK	LIM-domain kinase
MAPK	mitogen-activated protein kinase
MDA	microtubule disrupting agents
MDM2	murine double minute 2 (mdm2 oncogene)
MLC	myosin light chain
MLCK	myosin light chain kinase
MMTV	mouse mammary tumor virus
MMP	matrix metalloproteinases
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
N-WASP	N-Wiskott Aldrich syndrome protein
p130Cas	Crk-associated substrate
PBS	phosphate buffered saline
PI	propidium iodide
PI3K	phosphoinositide-3 kinase
PKB	protein kinases B
PKL	paxillin kinase linker
PLC	phospholipase C
PPR	proline-rich regions

PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate 1
Ras	Ras super family
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated kinase
qRT-PCR	quantitative real time- polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate - poly acrylamide gel electroforese
SF	scatter factor
SH	Src-homology domain
siRNA	short interference RNA
shRNA	short hairpin RNA
SRE	serum response element
PVDF	polyvinylidenefluoride
TAM	tumor-associated macrophages
TBP	0.05% Triton/0.5% BSA in PBS
TIRF	total internal reflection fluorescence
TRE	TPA response element

List of publications

1. Yafeng Ma, Bob van de Water and Sylvia Le Dévédec. Focal adhesion kinase inhibition as a potential strategy for anticancer therapies. (submitted to *Drug of the Future*)
2. Yafeng Ma, Saertje Verkoeijen, Maroesja J. van Nimwegen, John H. Meerman and Bob van de Water. Role of Fos related antigen-1 (Fra-1) in focal adhesion kinase (FAK) mediated chemoresistance in mammary adenocarcinoma tumors and lung metastases. Research paper (submitted to *Molecular Pharmacology*)
3. Yafeng Ma, Saertje Verkoeijen and Bob van de Water. c-Jun N-terminal kinase coordinates vincristine-induced Rho-kinase-dependent cell contractility through the focal adhesion-associated scaffold protein paxillin. Research paper (submitted to *Journal Pharmacology Experimental Therapeutics*)
4. Yafeng Ma, Hans de Bont, John H. Meerman and Bob van de Water. An autocrine CXCR3 activation-loop drives breast tumor cell migration and invasion through ERK and Akt signaling. Research paper (submitted to *Molecular Cancer Therapeutics*)
5. Yafeng Ma, Sylvia Le Dévédec, Saertje Verkoeijen and Bob van de Water. The Serine178 residue of paxillin determines EGF-induced cell migration of metastatic breast tumor cell MTLn3. (*In preparation*)

CURRICULUM VITAE

Yafeng Ma was born on January 28th, 1980 in HeNan, China. In 1997, she started her college education in Biochemistry department in Nanjing University (Nanjing, Jiang Su, China). In 2001, she received her bachelor degree and started her 3-year master study under supervision of Prof. Chen Junhui in Biochemistry and Molecular Biology (Biochemistry Dept., Life Science School) in the same university. Her research was about anti-tumor effects of extracts from human urine. From September 2004 till April 2009, she pursued her PhD project in Division of Toxicology in the Leiden/Amsterdam Center of Drug Research (Leiden University, The Netherlands) financed by a 4-year fellowship from the Chinese Scholar Council. Her research was under supervision of Prof. Bob van de Water and focused on focal adhesion-dependent signaling in breast cancer treatment. From May 2009, she is a postdoctoral researcher in the group of Prof. Laura Machesky at the Beatson Institute of Cancer Research in Glasgow, Scotland.